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BY

RUDOLPH J. ANDERSON, New Haven, LAFAYETTE B. MENDEL, New Haven, Conn.
Conn.

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ON THE CARBON DIOXIDE COMPOUNDS IN HEMOGLOBIN SOLUTIONS

BY O. M. HENRIQUES

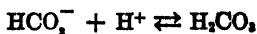
(From the Danish State Serum Institute, Copenhagen, Denmark)

(Received for publication, February 18, 1931)

In a recent number of this *Journal* Van Slyke and Hawkins (1930) have published some experiments on the velocity of the escape of CO_2 from hemoglobin- CO_2 solutions when exposed to reduced pressures as compared with the velocity of escape of CO_2 from salt solutions of similar buffer power. They undertook these experiments following the publication of preliminary notes of Henriques (1928). The results of more extended investigations of Henriques (1929) are not cited by Van Slyke and Hawkins.

Van Slyke and Hawkins confirm the experimental findings of Henriques that CO_2 escapes much more rapidly from hemoglobin solutions than from hemoglobin-free solutions when they are exposed to reduced pressure.

With the experimental results, however, the agreement between the authors ends. Henriques discussed two possibilities as the cause of the rapid evolution of CO_2 from hemoglobin solutions: (1) a sort of catalytic acceleration of the processes $\text{H}_2\text{O} + \text{CO}_2 \rightleftharpoons \text{H}_2\text{CO}_3$, and (2) formation of a complex molecular combination between hemoglobin and anhydrous CO_2 . As a result he came to the conclusion that catalysis seems improbable, while the existence of a complex molecular union was consistent with all existing experimental and physiological data. Van Slyke and Hawkins, on the other hand, come to the opposite conclusion, that a molecular combination seems improbable, while a sort of catalytic acceleration of the processes



by the erythrocyte contents seemed more likely.

They support their hypothesis by an experiment in which a

small amount of hemoglobin accelerated the escape of CO₂ almost as much as did large quantities.

It must be admitted that this experimental finding seems contrary to Henriques' hypothesis; nevertheless I think I am right, and Van Slyke and Hawkins incorrect, in the interpretation of experimental facts, and shall, therefore, present criticisms of their experiments as a quantitative test.

For a system of an aqueous phase in equilibrium with a CO₂-containing gas phase the following relation holds

$$\frac{[\text{H}_2\text{CO}_3]}{[\text{CO}_2] [\text{H}_2\text{O}]} = K_{\text{hydrate}}$$

or with the concentration of water = 1

$$(1) \quad \frac{[\text{H}_2\text{CO}_3]}{[\text{CO}_2]} = K_{\text{hydrate}}$$

$$(2) \text{ and } [\text{H}_2\text{CO}_3] = K_{\text{hydrate}} \cdot [\text{CO}_2]$$

The dissociation of the carbonic acid is governed by

$$(3) \quad \frac{[\text{H}^+] \cdot [\text{HCO}_3^-]}{[\text{H}_2\text{CO}_3]} = K_{\text{carbonic acid}}$$

Substituting [H₂CO₃] in Equation 3 by the expression from Equation 2, we derive the dissociation equation of CO₂

$$(4) \quad \frac{[\text{H}^+] \cdot [\text{HCO}_3^-]}{[\text{CO}_2]} = K_{\text{hydrate}} \cdot K_{\text{carbonic acid}} = K_1$$

K₁ is called the dissociation constant of CO₂.

The equations are valid only for infinite dilution. At finite dilutions we have to multiply the concentration terms by activity coefficients. Generally we calculate with the activity of the hydron and with concentration of the bicarbonate ions; the activity coefficients of the uncharged molecules of H₂CO₃ and CO₂ are taken as equal to 1. Equation 4, therefore, is transformed to

$$(5) \quad \frac{a[\text{H}^+] \cdot [\text{HCO}_3^-] \cdot f_a}{[\text{CO}_2]} = K_1 \text{ and } \frac{a[\text{H}^+] \cdot [\text{HCO}_3^-]}{[\text{CO}_2]} = \frac{1}{f_a} \cdot K_1 = K'_1$$

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THE EFFECT OF ULTRA-VIOLET IRRADIATION UPON THE FREE STEROLS OF LANOLIN

BY ADOLPH BERNHARD AND I. J. DREKTER

(From the Achelis Laboratory and A. Jacobi Division for Children, Lenox
Hill Hospital, New York)

(Received for publication, June 11, 1931)

It has been shown that following lanolin inunctions and ultra-violet irradiation there is a rise in the blood sterol (cholesterol) of children and adults (1, 2). Further investigation of the nature of this rise in sterol content after ultra-violet irradiation was undertaken, and the observations are reported in this paper.

Lanolin consists of different fatty acids, cholesterol, cholesterol esters, oxysterol, so called isocholesterol, and agnosterol and lanosterol, the latter two not being precipitated by digitonin (3). In addition to these, lanolin contains other alcohols, such as lanolin alcohol, carnaubyl, ceryl, and undetermined higher alcohols, all of which make up about 50 per cent of lanolin. The total sterol content, after saponification, is about 20 per cent, and the free sterols which are precipitated by digitonin, calculated as free cholesterol, are about 0.9 per cent.

EXPERIMENTAL

Anhydrous lanolin (U.S.P.)¹ was spread out in a thin layer in an evaporating dish and irradiated with a Hanovia quartz mercury vapor lamp at a distance of 12 inches for different time periods. During the irradiation, the lanolin which melts is thoroughly and constantly stirred. The intensity of the ultra-violet energy produced by the lamp was checked biweekly by the method of Anderson and Robinson (4).

After the observed time of irradiation of the lanolin, 100 mg. were transferred to a Pyrex extraction flask with 100 cc. of hot

¹ The lanolin used in these experiments was obtained from Pfaltz and Bauer.

95 per cent alcohol. 3 cc. of a 1 per cent digitonin solution were added and the mixture refluxed for 1 hour on a boiling water bath. It was then allowed to stand at room temperature overnight. The precipitated sterols were filtered through a weighed Jena fritted glass filter crucible (No. 4 porosity), then washed thoroughly with cold alcohol and with an excess of ether to insure complete removal of any fats insoluble in cold alcohol. The crucibles were dried at 80° for 15 minutes, placed in a desiccator for $\frac{1}{2}$ hour, and then weighed, the Bunge micro balance being used. The weight of the precipitated sterols when divided by 4, indicates the free sterols expressed as free cholesterol. All determina-

TABLE I
Free Sterols Precipitated by Digitonin after Ultra-Violet Irradiation

Time of irradiation	Sterols precipitated by digitonin expressed as free cholesterol per 100 mg lanolin
<i>min.</i>	<i>mg.</i>
0	0.93
2	1.92
4	2.57
6	3.22
8	3.22
10	3.45
15	4.00
30	5.00
60	5.37
120	5.55

tions were made in duplicate, while those made on untreated lanolin were in quadruplicate. The average of each group is given in Table I.

DISCUSSION

Before irradiation with ultra-violet light, the free sterol of lanolin which is possible of precipitation with digitonin when expressed in terms of free cholesterol is 0.93 mg. per 100 mg. of lanolin. After irradiation, there is a gradual progressive increase in the amount of free sterols precipitable by digitonin, the maximum being reached at the end of 1 hour. After 2 hours irradiation, there is no further significant increase. The reaction does not seem to be reversible, since 3 weeks after irradiation the amount

of sterols precipitated by digitonin remained unchanged. Color reactions for ergosterol (5) in the lanolin both before and after irradiation were not specific, a brownish green color of the same intensity being obtained in both instances.

Attempts were made to isolate cholesterol from lanolin which had been irradiated for 1 hour. The crystalline fractions obtained could not be identified as pure cholesterol. It is evident that digitonin precipitates substances other than cholesterol. This observation has also been noted by Anderson (6), who obtained substances from normal dog plasma which were precipitated by digitonin, but were not cholesterol.

SUMMARY

1. The amount of free sterols in anhydrous lanolin which is precipitated by digitonin, and expressed in terms of free cholesterol, is 0.93 mg. per 100 mg. of lanolin.

2. When anhydrous lanolin is irradiated with ultra-violet light, there is a progressive gradual rise in the amount of free sterols precipitated by digitonin, the maximum rise being reached at the end of 1 hour. The free sterols expressed as free cholesterol increased from 0.93 to 5.37 mg. per 100 mg. of lanolin.

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THE EQUILIBRIUM BETWEEN CEREBROSPINAL FLUID AND BLOOD PLASMA

VI. THE DISTRIBUTION OF SODIUM BETWEEN CEREBROSPINAL FLUID AND BLOOD SERUM

By MARY ELIZABETH DAILEY

*(From the Department of Neuropathology, Harvard Medical School, and the
Neurological Unit, Boston City Hospital, Boston)*

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There are few data in the literature on the distribution of sodium between serum and cerebrospinal fluid. Mestrezat (1) determined the concentration of individual inorganic elements on a mixture of cerebrospinal fluid from twenty normal individuals and obtained a value of 322 mg. per 100 cc. for sodium.

Pincus and Kramer (2) and Weston and Howard (3) have published short series of determinations made on samples of blood and spinal fluid taken at the same time. Pincus and Kramer state that the sodium concentration of spinal fluid is essentially the same as that of plasma although in nine out of eleven cases they found the same relationship that we consistently found, namely, slightly higher values for sodium in cerebrospinal fluid. Their figures in eleven instances average 331 mg. per 100 cc. for serum and 344 mg. per 100 cc. for cerebrospinal fluid. Weston and Howard in a series of twenty-seven cases of manic-depressive insanity obtained an average value of 331 mg. per 100 cc. for both serum and cerebrospinal fluid, with a range of 297 to 343 mg. per 100 cc. in serum and 303 to 346 mg. per 100 cc. in cerebrospinal fluid. Leipold (4), quoted by Walter (5), gives values of 257 to 331 mg. of sodium per 100 cc. for spinal fluid and found the sodium value in the serum equal to that in the spinal fluid.

Depisch and Richter-Quittner (6) have studied the inorganic elements in serum and cerebrospinal fluid. They have reported such extraordinary values, especially for calcium, as to cast serious doubt on the accuracy of the determinations reported.

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We are here reporting the results of simultaneous sodium and chloride determinations on blood serum and cerebrospinal fluid in 97 instances. The complete data on most of these cases, with diagnosis where ascertained, is reported in Papers I and II of this series (7, 8) and the case numbers used in this paper correspond to those of the earlier papers.

Methods of Procedure

The actual values reported in this paper were determined by the following methods: chlorine by the Wilson and Ball modification of the Van Slyke method (9), and sodium by the Rourke modification of the Kramer-Gittleman method (10).

TABLE I
Sodium in Serum and Body Fluids

Sample	Original value	Final value	Na added	Na recovered
	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
Serum	308	336	28	28
	308	336	28	28
Chest fluid	306	334	28	28
	306	336	28	30
Cerebrospinal fluid	330	348	17	18
	330	347	17	17
	330	345	17	15

We have made the following tests upon the accuracy of the sodium method. Two solutions were made from Kahlbaum's best grade sodium chloride to contain 936 and 2552 mg. of NaCl per 100 cc. respectively. For the sodium determination on the first solution 1 cc. samples were used and values of 368 and 369 mg. per 100 cc. were obtained. Chloride determined was 567 mg. per 100 cc. Both sodium and chloride values correspond to NaCl solution of 936 mg. of NaCl per 100 cc. For the determination on the more concentrated solution 0.5 cc. samples were used for sodium determination and values of 1005, 998, and 1002 were obtained, averaging 1001; values for chloride were 1550, 1549, and 1544, averaging 1548 mg. per 100 cc. The actual sodium in the solution was 1003 and chloride 1549 mg. per 100 cc. These results

demonstrate the absolute accuracy of the methods for sodium and chloride and the fact that 5 mg. of sodium can be accurately determined with the solutions used.

TABLE II
Cases with Normal Serum Chlorides, Freezing Points Identical

Case No.	Sodium		Chloride		Ratio	
	Serum	Spinal fluid	Serum	Spinal fluid	Spinal fluid Na Serum Na	Serum Cl Spinal fluid Cl
	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.		
1	317	331	345	425	1.04	0.81
3	324	334	355	439	1.03	0.80
4	316	324	372	447	1.03	0.83
6	303	306	348	438	1.01	0.79
7	314	321	362	439	1.02	0.82
8	322	324	351	436	1.01	0.81
9	311	324	359	441	1.04	0.81
10*	335	343	375	452	1.02	0.83
12	315	328	359	439	1.04	0.82
38	317	326	371	438	1.03	0.85
39	335	342	375	450	1.02	0.83
41	309	323	349	435	1.05	0.80
42	315	319	362	438	1.01	0.83
43	308	324	358	436	1.05	0.82
44	307	313	360	445	1.02	0.81
73	316	318	378	447	1.01	0.85
78	315	324	351	431	1.03	0.81
96	302	301	348	418	1.00	0.83
119	323	339	364	439	1.05	0.83
121	305	309	354	430	1.01	0.82
126	330	343	364	439	1.04	0.83
222	327	333	350	439	1.02	0.82
326	311	313	348	431	1.01	0.81
High.....	335	343	375	452	1.05	0.85
Low.....	302	301	345	418	1.00	0.79
Average...	316	324	359	438	1.03	0.82

* Fluid from cisterna magna.

The sodium contents per 100 cc. of serum, chest fluid, and cerebrospinal fluid samples were next determined and then increased by mixing with an equal volume of NaCl solution contain-

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ing 364 mg. of sodium per 100 cc. and the added amounts were recovered as shown in Table I.

These very satisfactory results assured us that the method was reliable in our hands both for serum and cerebrospinal fluid. The

TABLE III
Cases with Normal Serum Chloride, Freezing Points Not Identical

Case No	Sodium		Chloride		Ratio	
	Serum	Spinal fluid	Serum	Spinal fluid	Spinal fluid Na Serum Na	Serum Cl Spinal fluid Cl
	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>		
46	334	346	373	449	1.04	0.83
47	305	297	369	449	0.97	0.82
48	314	327	364	439	1.04	0.83
49	320	304	354	444	0.95	0.80
50	314	315	356	444	1.00	0.80
51	314	324	358	439	1.03	0.82
53	322	331	375	441	1.03	0.85
54	321	329	372	447	1.02	0.83
55	335	348	365	444	1.04	0.82
58	305	316	354	441	1.04	0.80
59	342	348	362	438	1.02	0.83
74	321	325	364	439	1.01	0.83
75	323	326	356	436	1.01	0.82
115	324	326	345	441	1.01	0.78
123	304	317	364	441	1.04	0.83
215	294	321	356	433	1.09	0.82
327	311	331	367	441	1.06	0.83
330	321	327	378	413	1.03	0.92
331	295	305	358	450	1.03	0.80
113	321	325	380	441	1.00	0.86
112	297	306	378	444	1.03	0.85
98	311	316	354	431	1.02	0.82
High.....	342	348	380	450	1.09	0.92
Low.....	294	297	345	413	0.95	0.78
Average..	316	323	364	441	1.02	0.82

results reported in this paper for sodium are average values of duplicate determinations upon 1 cc. of serum or cerebrospinal fluid which checked within 2 per cent, generally within 1 per cent.

Results

Serum Sodium—Our values for serum sodium in 85 cases, Tables II to VII, including twenty-eight cases of meningitis, range from

TABLE IV
Cases with Low Serum Chlorides, Freezing Points Identical

Case No.	Sodium		Chloride		Ratio	
	Serum	Spinal fluid	Serum	Spinal fluid	Spinal fluid Na Serum Na	Serum Cl Spinal fluid Cl
	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.		
157	306	317	341	420	1.06	0.81
175	330	337	336	424	1.02	0.79
187	274	288	333	383	1.05	0.87
219	260	286	299	406	1.10	0.74
High.....	330	337	341	424	1.10	0.87
Low.....	260	286	299	383	1.02	0.74
Average..	292	307	327	408	1.06	0.80

TABLE V
Cases with Low Serum Chlorides, Freezing Points Not Identical

Case No.	Sodium		Chloride		Ratio	
	Serum	Spinal fluid	Serum	Spinal fluid	Spinal fluid Na Serum Na	Serum Cl Spinal fluid Cl
	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.		
155	285	322	343	423	1.13	0.81
160	331	344	340	423	1.04	0.80
197	298	308	312	401	1.03	0.78
208	319	327	333	388	1.03	0.86
216	305	315	319	383	1.03	0.83
220	312	326	302	427	1.04	0.71
156	313	343	341	436	1.10	0.78
192	312	304	325	388	0.97	0.84
High.....	331	344	344	436	1.13	0.86
Low.....	285	304	302	383	0.97	0.71
Average..	307	322	330	414	1.05	0.80

260 to 348 with an average of 311 mg. per 100 cc. Our most normal serum values, forty-two cases (from patients who had fasted

TABLE VI
Cases with Meningitis

Case No.	Sodium		Chloride		Ratio	
	Serum	Spinal fluid	Serum	Spinal fluid	Spinal fluid Na Serum Na	Serum Cl Spinal fluid Cl

Colon bacillus meningitis (one patient)						
	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.		
298	300	302	349	410	1.01	0.85
299	284	289	316	378	1.02	0.84
300	295	310	318	383	1.05	0.83
301	303	311	335	383	1.03	0.87
302	302	319	319	410	1.06	0.78
303	311	317	339	408	1.02	0.83
304	315	314	345	420	1.00	0.82
305	317	313	354	430	0.99	0.82
306	284	307	338	407	1.08	0.83
307	309	309	330	383	1.00	0.86
308	318	318	348	420	1.00	0.83
309	314	316	341	428	1.01	0.80
310	300	311	348	413	1.03	0.84

Acute purulent meningitis						
325	332	343	355	422	1.03	0.84
272	299	307	303	382	1.03	0.80
279	329	317	341	393	0.96	0.87
273	296	303	322	393	1.02	0.82
278	323	341	372	410	1.06	0.91
288	298	300	308	364	1.01	0.85
290	295	306	301	351	1.04	0.86
332*	348	371	414	481	1.07	0.86
280	329	334	341	404	1.02	0.84
323	290	305	339	395	1.05	0.86

Tuberculous meningitis						
345	274	279	289	335	1.03	0.86
266	282	304	314	345	1.08	0.91
334	309	320	298	362	1.04	0.82
333	315	332	308	367	1.05	0.84
335	306	313	290	356	1.02	0.81
High.....	332	343	372	430	1.08	0.91
Low.....	282	289	289	335	0.96	0.78
Average..	305	313	323	390	1.03	0.84

* A case of meningitis following brain abscess, which was not included in the average as the sodium and chloride values are far above those in all other cases.

12 hours or longer) with normal serum chloride (345 to 378 mg. per 100 cc.)¹ as recorded in Tables II and III, range from 294 to 342 mg. per 100 cc. and average 316. These values are somewhat lower than those reported by Rourke (10) on fourteen normal subjects, namely 330 to 347 mg. per 100 gm. equivalent to 338 to 357 mg. per 100 cc.

In Tables IV and V, twelve cases with low serum chloride values, the range for serum sodium is 260 to 331 mg. per 100 cc. with an average of 305. This is distinctly lower than the average for

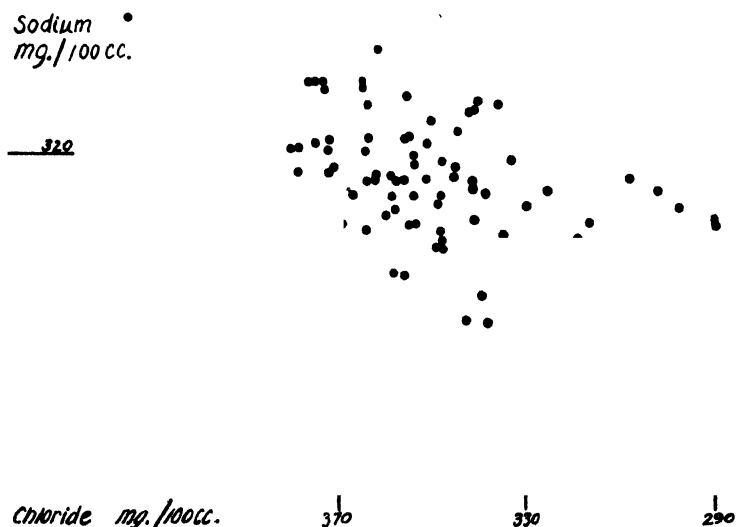


CHART 1. This chart shows the tendency for the serum sodium to vary directly with the serum chloride.

Tables II and III with normal serum chlorides and indicates that low serum sodium values are associated with low chlorides. The low average for serum sodium, 305 mg. per 100 cc., in the twenty-seven meningitis cases recorded in Table VI, is further evidence of this relationship between serum sodium and chloride levels. In Chart 1 all the data on serum sodium are plotted against the corresponding serum chloride values.

¹ The freezing point data on these cases, as well as non-protein nitrogen, sugar, spinal fluid pressure, cell count, and gold sol reaction may be found in the earlier papers (7, 8).

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Spinal Fluid Sodium—Our values for spinal fluid sodium are slightly lower than those reported by Pincus and Kramer (2) or by Weston and Howard (3). The range for the 85 cases is 279 to 348 with an average of 320 mg. per 100 cc. The range in Tables II and III, the most normal cases, is 297 to 348 mg. per 100 cc., with an average of 324 mg. per 100 cc. almost identical with the value 322 mg. per 100 cc. obtained by Mestrezat (1). The average for the twelve cases with low serum chlorides in Tables IV and V is

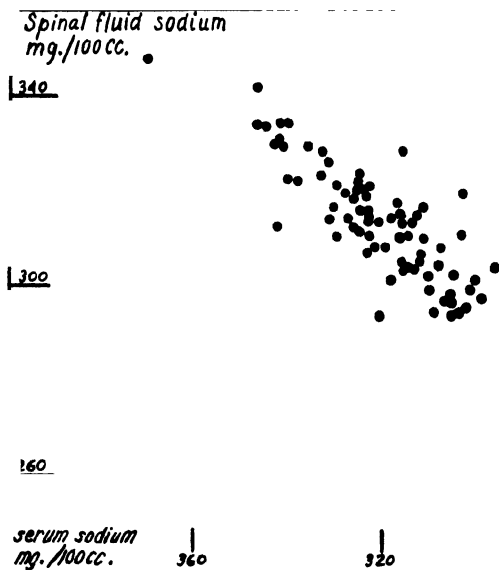


CHART 2. This chart shows the very definite variation of spinal fluid with change in serum sodium; *i.e.*, the constancy of the ratio of serum to spinal fluid sodium.

317 mg. per 100 cc. The average value for sodium in the spinal fluid in twenty-seven cases of meningitis is 313 mg. per 100 cc. Therefore, in meningitis low sodium values are associated with low chloride values in the cerebrospinal fluid as well as in the serum.

Distribution of Sodium between Serum and Cerebrospinal Fluid—The sodium value in spinal fluid is nearly always slightly greater than that in the serum (in only six instances was the reverse true). The ratio of spinal fluid sodium to serum sodium ranges between

1.00 and 1.05 in forty-eight of the 57 non-meningitic cases. Chart 2 shows the tendency for the spinal fluid sodium to vary directly with the serum sodium.

In ten instances a dilution of the blood serum was produced by water drinking during the antidiuretic action of posterior pituitary

TABLE VII
*Changes in Sodium and Chloride Values of Serum and Cerebrospinal Fluid with Acute Blood Dilution**

Case No.		Chloride		Sodium	
		Serum	Spinal fluid	Serum	Spinal fluid
		mg per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
119	Before water	364	439	323	339
	After "	330	423	303	314
126	Before "	364	439	330	343
	After "	335	414	303	328
78	Before "	351	431	315	324
	After "	322	407	286	308
4	Before "	372	447	316	324
	After "	344	441	302	321
59	Before "	362	438	342	348
	After "	337	419	308	331
53	Before "	375	441	322	331
	After "	354	436	310	332
41	Before "	349	435	309	323
	After "	336	431	302	321
74	Before "	364	439	321	325
	After "	354	423	306	320
47	Before "	369	449	305	297
	After "	362	445	290	294
75	Before "	356	436	323	326
	After "	351	417	313	315
54	Before saline	372	447	321	329
	After "	362	441	307	324
3	Before water	353	439	324	334
	After "	359	439	324	337

* The details of these experiments may be found in Paper V of this series (11).

lobe extract (β -hypophamine). In these patients both serum chloride and sodium showed a definite drop as the result of the blood dilution. This dilution was reflected in the cerebrospinal fluid chloride and sodium values, though often to a less degree.

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In Table VII these cases are arranged in order of decreasing change in serum chloride. In Case 54 a similar dilution was produced by intravenous injection of hypotonic Ringer's solution. Case 3 is a control case where water was given without vasopressin, and no blood or spinal fluid dilution took place. These experiments

Sodium Decrease
mg./100 cc.

Chloride decrease
mg./100 cc.

CHART 3. This chart shows the proportional decrease in serum sodium with decrease in serum chloride in acute experiments and the decrease in spinal fluid sodium accompanying the consequent decrease in spinal fluid chloride. The cases here charted are those outlined in Table VII. The solid circles represent serum values; the clear circles, values for cerebrospinal fluid.

prove that acute changes in the sodium and chloride level in the blood are reflected in the cerebrospinal fluid (Chart 3). The sodium values change proportionally with change in chloride in these experiments.

Tables II to VII give the ratios for serum to spinal fluid chloride and spinal fluid to serum sodium as actually determined. In a

paper now in preparation (12) we will discuss the distribution of sodium between serum and cerebrospinal fluid as related to the Donnan membrane equilibrium.

SUMMARY

Data are presented on sodium and chloride values in human serum and cerebrospinal fluid in 97 instances.

The serum sodium value tends to vary directly with the serum chloride.

The spinal fluid sodium is, in most instances, slightly higher than that of serum.

In meningitis the average values for sodium in both serum and cerebrospinal fluid are slightly lower than in our normal group. The lowest values tend to be associated with low chloride values, although the sodium diminution in serum and cerebrospinal fluid is usually less marked than the diminution in chloride.

Acute changes in the sodium and chloride content of serum by blood dilution are reflected in the spinal fluid, and the change in sodium is proportional to the chloride change.

The average ratio of spinal fluid sodium to serum sodium is 1.03.

The average ratio of serum chloride to spinal fluid chloride is 0.82.

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THE RELATIVE COMPOSITION OF SEA WATER AND OF THE BLOOD OF *LIMULUS POLYPHEMUS*

By MARY ELIZABETH DAILEY, FRANK FREMONT-SMITH, AND
MARGARET P. CARROLL

(From the Department of Neuropathology, Harvard Medical School, Boston,
and the Marine Biological Laboratory, Woods Hole)

(Received for publication, June 5, 1931)

In 1929 Thomas (1) investigated the effect of hemocyanin on the distribution of chloride between sea water and the blood of *Limulus polyphemus*. He concluded that "The Donnan equilibrium due to the hemocyanin of the serum will account theoretically for a considerable part of the difference in chloride concentration between the serum and sea water."

We have made comparative determinations of freezing point, sodium, chloride, total nitrogen, non-protein nitrogen, sugar, total solids, and specific gravity on *Limulus* serum and sea water in eleven instances in order to obtain further evidence of the isotonicity of the serum of *Limulus* with sea water, and to see whether or not the distribution of sodium as well as chloride is in accordance with the Donnan theory.

Method of Procedure

Samples of blood were obtained by inserting a needle into the body cavity at the attachment of the caudal spine. The blood was centrifuged and all determinations were made on serum. The animals were brought to the laboratory in sea water from the bay in which they had been kept for several weeks. This sea water was analyzed as representing the medium with which the animals should be in equilibrium.

The freezing point was determined by the method of Beckmann (2), a Heidenhain thermometer being used; sugar by the method of Folin and Wu (3), with the modified sugar tubes described by Rothberg and Evans (4); non-protein nitrogen by the Kjeldahl

method (5) on 200 cc. of Folin-Wu filtrate in the first three instances, and subsequently by the micro method of Folin and Wu (6), 10 cc. of filtrate being used. Chlorides were determined by the Wilson and Ball (7) modification of the Van Slyke method, 4 cc. of 0.15 N silver nitrate solution being used per cc. of serum or sea water. Sodium was determined on 0.4 cc. samples by the Rourke (8) modification of the Kramer-Gittleman method after it had been demonstrated that the reagents used were adequate for the determination of 5 mg. of sodium (9). Total solids were determined by drying to constant weight at from 105–110°, protein nitrogen by the Kjeldahl method (5), and specific gravity with a 10 cc. Gay-Lussac specific gravity bottle.

Results

The data are presented in Table I. The reducing substances in the blood are seen to vary from 11 to 22 mg. of glucose per 100 cc. Experiments showing the results of yeast fermentation and hydrolysis on the serum reducing substances of *Limulus polyphemus* and certain elasmobranchs will be reported later. In these experiments we have shown that these reducing substances in the blood of *Limulus polyphemus* are completely fermentable by yeast, not increased by hydrolysis, and are therefore probably glucose.

In seven out of nine instances the serum and sea water are isotonic within 0.01°, the greatest difference being 0.02°. This is in agreement with the findings of Duval (10) working with other marine invertebrates.

The chloride content of the sea water is greater than that of serum in all but one case, in which the values are identical. The average ratio for serum chloride to sea water chloride is 0.964 and is in close agreement with the average ratio of 0.953 obtained by Thomas (1). The sodium value in the serum is greater than that in the sea water in every instance. The average ratio for sea water sodium to serum sodium is 0.989.

By means of the data on total solids and specific gravity we have calculated the water content of *Limulus* serum and of sea water, the sodium and chloride values in water content, and the corresponding ratios. These are shown in Table II. For the calculation of the theoretical Donnan ratio we assumed the same

values for per cent nitrogen in hemocyanin (11) and for base bound by protein (12) as were used by Thomas (1). The equation described by Thomas (1) for the Donnan ratio is

$$r = \frac{(\text{Cl}^-)_{\text{se}} - \frac{1}{2}(\text{BHcy})_s}{(\text{Cl}^-)_{\text{se}}}$$

It will be seen from Table II that the agreement of the chloride ratio (0.998) with the theoretical ratio (0.992) is well within the

TABLE III
*Composition of Sea Water**

Date	Sodium	Chloride	Specific gravity	Freezing point depression	Total solids
1929	mg per 100 cc.	mg. per 100 cc.		°C.	gm. per 100 gm
July 8		1804			
" 9		1805			
" 15	955	1814			
" 17	978				
" 18	974				
" 20	967	1808		1.831	
" 23			1.0245	1.831	3.36
" 24			1.0243	1.813	
" 25	1016	1807			
" 29				1.812	
" 30	1028	1823	1.0242	1.798	3.33
Aug. 13	1028	1829			
" 16	1021	1853	1.0243	1.797	3.44
" 21	1023	1853	1.0243	1.799	3.43
" 24	1029	1832	1.0246	1.793	
" 26	993	1821	1.0232	1.769	
" 29		1821	1.0241	1.793	

* The sea water was obtained from water continuously pumped from the bay except on July 30 and August 16 when it was taken from the open sea, and on August 21 and thereafter when it was taken directly from the bay near the shore.

error of the experiments (0.6 per cent). The agreement of the sodium ratio, while not so good, is within 2 per cent of the theoretical ratio.

Effect of Rainfall upon Composition of Sea Water—During the summer of 1929 we had an opportunity to observe a dilution of the sea water at Woods Hole, as the result of a heavy rain-storm.

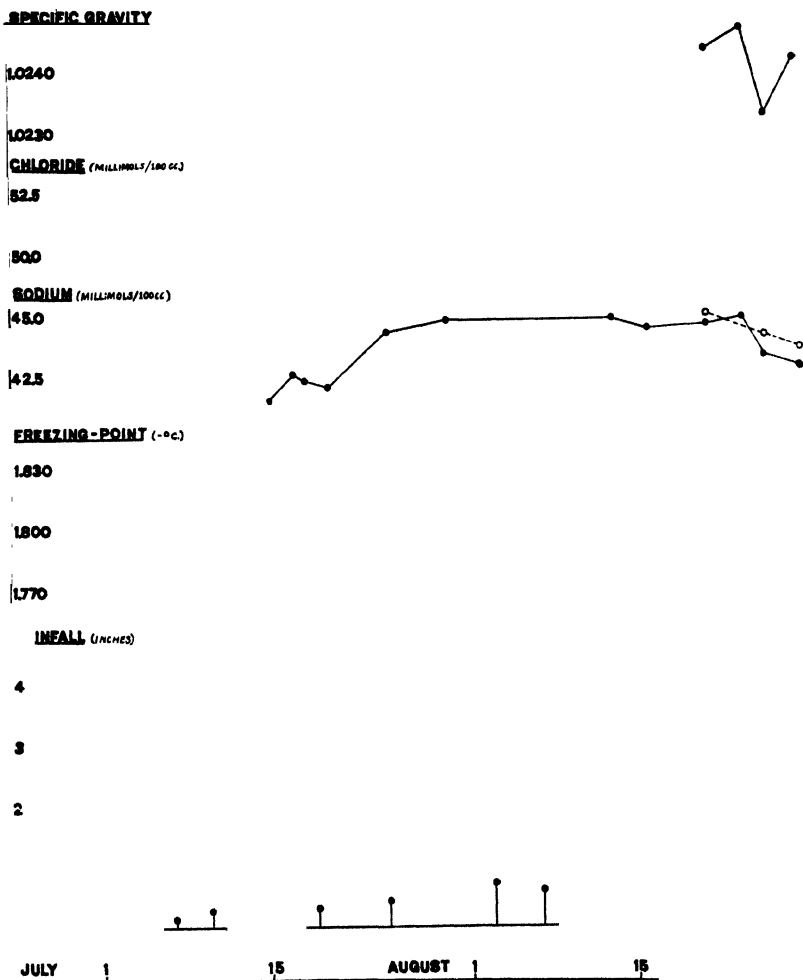


CHART 1. The influence of heavy rain-storm on the composition of sea water and of the blood of *Limulus polyphemus*. The dots indicate sea water and the circles *Limulus* blood serum.

From July 8 to August 29 we made determinations on the composition of sea water on 17 days. These data, together with the precipitation¹ during this period, are shown in Table III and

¹ Data on the rain precipitation at Woods Hole were obtained from the Blue Hill Observatory, Boston, Massachusetts.

Chart 1. Up to August 23 there was very little rainfall. The minor variations in the composition of the sea water during this period are due to unknown causes, possibly to variations in the composition of water flowing from the shore into the sea. On August 23 a heavy rainfall, precipitation of 4.85 inches, occurred. This resulted in an appreciable dilution of the sea water which was first shown on August 24, the day after the storm, but was more marked August 26, 2 days later. This delayed dilution probably results chiefly from rain water flowing off the land into the sea rather than from that falling directly into the sea.

On August 21, 24, and 26 we also analyzed the serum of *Limulus polyphemus*. The average values for the three specimens determined on each of these days are plotted in Chart 1. This shows that the dilution of the sea water resulting from the rain-storm was reflected in the blood of *Limulus polyphemus*, and emphasizes the importance of comparing the blood with sea water each time equilibrium studies are made. The maintenance of isotonicity of *Limulus polyphemus* serum with sea water when the sea water is diluted by rain is similar to that observed by Duval (10) in his work on the relationship between the internal and external environments of aquatic animals. He diluted sea water and found the blood of marine invertebrates which he studied to become correspondingly dilute. *Limulus polyphemus* was not included in his series.

SUMMARY

The blood serum of *Limulus polyphemus* is isotonic with sea water. This isotonicity is maintained when the sea water becomes appreciably diluted by a heavy rain-storm. The distribution ratio for chlorine between sea water and the serum of *Limulus polyphemus* closely approximates the theoretical Donnan ratio. The sodium ratio agrees with the theoretical ratio within 2 per cent.

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A NEW COLOR REACTION FOR SOLUBLE ORGANIC SULFUR COMPOUNDS*

By IRVINE W. GROTE

(From the Research Laboratories of Parke, Davis and Company, Detroit)

(Received for publication, June 22, 1931)

In view of the increasing importance of reduced sulfur compounds in biological chemistry, a study of the nitroprusside reaction was recently made in this laboratory in an attempt to render the reaction more specific for compounds of the type $C-S-H$, since sodium nitroprusside in alkaline solution not only gives coloration with compounds of this type but also with ketones, aldehydes, creatinine, thio ethers, and many alkaloids. Such interference was found to be practically eliminated by working at pH 8 to 9 with saturated sodium bicarbonate as the alkaline agent in place of the more generally used carbonate or hydroxide. In bicarbonate solution, the immediate coloration from ketones, aldehydes, etc. is so slight that the test for $C-S-H$ may be carried out in aqueous acetone solution without interference. With potassium cyanide being used as a reducing agent, the test can also be applied to compounds of the disulfide type $C-S-S-C$.

During the course of this work the observation was made that sodium nitroprusside solution previously exposed to light for some time gives an intense blue color with compounds of the thiourea type, in addition to the normal purple-red color with $C-S-H$. A search of the literature revealed that while Hofmann¹ noted a blue color as an intermediate in the formation of the red compound, $Na_2Fe(CN)_5NOSCNHNH_2$, from thiourea and ordinary nitroprusside, we were unable to find further mention of a blue coloration.

Sodium nitroprusside solution after several days exposure to indirect light, or a few hours to direct sunlight, changes from a

* This paper is published as Reprint 451.

¹ Hofmann, K. A., *Ann. Chem.*, **312**, 28 (1900).

cherry-red color to yellow, then green, and finally Prussian blue coagulates out and the solution may be filtered to yield a yellow-brown filtrate. This solution gives an intense blue color in bicarbonate solution with compounds of the thiourea type as well as the normal purple-red with $C-S-H$. During the reaction in the light a strong odor of cyanide is present and bubbles of nitrogen are given off. No further reaction apparently takes place upon further exposure to light for short periods. If placed in the dark without filtration for a few weeks, the light-treated solution will go back to the original cherry-red color and no longer give the new reaction with $C=S$ but will again do so after reexposure to light.

The rate of this photochemical change may be observed by adding a small amount of thiourea to fresh sodium nitroprusside solution and noting the development of the blue coloration upon exposure to light. If a slight amount of sodium bicarbonate is added, the reaction is photochemically reversible, the solution turning blue in the light and crimson when then placed in the dark. This alternate change of color with light and darkness may be repeated a dozen or more times if free access to air is allowed. A further study of this interesting reversible reaction will be reported upon in a later publication.

A number of derivatives of sodium nitroprusside were prepared in an attempt to duplicate the action of sunlight. The procedures of Hofmann were followed.²

Reduction of sodium nitroprusside in alkaline solution by means of hydroxylamine hydrochloride yields a yellow compound, sodium aquoferrocyanide, $Na_3Fe(CN)_5 \cdot H_2O$. This compound fails to give either the usual nitroprusside reaction or the new reaction with thiourea.

Treatment of this yellow compound with bromine water in neutral solution gives an intense purple compound to which Hofmann assigns the formula $Na_2Fe(CN)_5 \cdot H_2O$ (sodium aquoferri-cyanide). This compound was found to give an intense blue with thiourea after several minutes, more quickly on warming, but not as promptly as the material prepared by sunlight. The intense color of the reagent was objectionable also.

On standing in solution for some time in the presence of sodium bicarbonate, the purple color of sodium aquoferri-cyanide is

² Hofmann, K. A., *Ann. Chem.*, **312**, 1 (1900).

changed to a dark yellow-brown. This yellow compound reacts instantly with thiourea to give a blue color but does not give the usual nitroprusside reactions with mercaptans, etc.

Preparation of Test Solution for $C=S$, $C-SH$, and $C-S-S-C$ Compounds

A satisfactory test solution giving both the new reaction with $C=S$ compounds and the usual nitroprusside reactions may be prepared as follows: 0.5 gm. of sodium nitroprusside (sodium nitroferri-cyanide) is dissolved in 10 cc. of water at room temperature, 0.5 gm. of hydroxylamine hydrochloride is added, followed by 1 gm. of sodium bicarbonate. After evolution of gas has ceased, 2 drops of bromine are added. Excess bromine is removed by aeration and the dark greenish or black-brown solution filtered and made up to 25 cc. This solution behaves like that prepared by exposure of sodium nitroprusside to sunlight and contains a mixture of several compounds, one of which reacts with the thiourea type and another like ordinary nitroprusside. The solution is stable for about 2 weeks, gradually losing reactivity toward $C=S$. No further purification is necessary for general test use.

A black, hygroscopic compound, yellow in dilute solution and reacting instantly with thiourea with formation of an intense blue, can be separated from the solution prepared as above after several precipitations from aqueous solution by addition of an alcohol-ether mixture. The ordinary nitroprusside reactions are not given by this purified material, mercaptans in alkaline solution giving only a transient light green color, quickly turning yellow. The purified reagent offers no especial advantages over the crude solution and is much less stable. The active compound has not yet been identified.

The alcohol-ether filtrates discarded in this purification process, in addition to a small amount of the black compound and another compound reacting like ordinary nitroprusside, contain still another compound giving a blue coloration with $C=S$ on slight warming or more slowly at room temperature. This reacts differently from that of the active alcoholic precipitant in that the blue coloration starts at the solution surface and works down while the former gives color throughout almost instantly. In

addition, the blue color obtained with the filtrate and thiourea in bicarbonate solution when allowed to stand for a few hours, or more rapidly at 50–60°, changes through a purple-red to an intense crimson which turns blue in slightly acid solution and again red when made weakly alkaline. This behavior of forming the blue to red coloration with thiourea is also shown by the general test reagent prepared above and that prepared by action of light upon nitroprusside but is not a property of the purified alcoholic precipitant. The preliminary blue color formed in alkaline solution does not show a color change when made slightly acid.

Method of Test—5 to 20 mg. of the compound to be tested are dissolved in 2 to 3 cc. of water and solid sodium bicarbonate added to excess. About 0.5 cc. of the general test reagent is then added. Purple-red given instantly or within 10 minutes indicates C–S–H while an intense green or blue indicates C=S or E=S (where E is any single non-metallic element). Both colors may fade more or less rapidly but often reappear upon addition of fresh reagent. If no color appears within 10 minutes an equal volume of 5 per cent potassium cyanide solution is added. C–S–S–C compounds will give a pink to purple-red within $\frac{1}{2}$ hour. Ring-linked sulfur compounds of both the C–S–H and C–S–S–C type may fail to react, such as diparatolyl disulfide. Organo metallic compounds such as the type C–S–Bi=R₂ may require a preliminary warming with dilute ammonia before reacting.

The color with the type C=S is usually first green, then turquoise, and finally deep blue. In some cases the color stops at the deep green stage, in others at the turquoise. In a few cases, as with thiourea previously mentioned, it may go through the blue stage to a purple-red and finally crimson after several hours. The test for C=S cannot be carried out in acid solution as the reagent is decomposed with liberation of Prussian blue.

The blue color is given in bicarbonate solution with the new reagent not only by compounds of the type C=S but also by N=S, S=S, and probably in general by the type E=S, where E is any single non-metallic element doubly linked to divalent sulfur. Potassium thiocyanate, of the type N=S, and sodium thiosulfate, of the type S=S, give the reaction beautifully.

The blue coloration in many cases is sufficiently stable for colorimetric quantitative assay. Care must be taken to provide

dilutions sufficient to prevent maximum color formation as the reaction is extremely sensitive. Distinct green coloration is given by thiourea 1 to 1 million and excellent quantitative comparisons can be made 1:100,000 to 1:200,000. Sodium thiosulfate is best matched 1:1000 to 1:5000, the blue color in this case remaining stable for days.

Some compounds destroy the reagent but many of these give transient colors of the correct type. Thioacetic acid, which attacks the reagent, gives a flash of blue, indicating the formula CH_3CSOH rather than CH_3COSH . Compounds of the thiophenol type give only a fugitive purple-red.

If both $\text{C}=\text{S}$ and $\text{C}-\text{S}-\text{H}$ are present, the blue color alone is produced but fades rapidly. In this case more stable color is

given in neutral solution. Potassium xanthate $\text{O}-\text{C}(\text{S})_2\text{SK}$ gives a blue with the new reagent as well as with ordinary sodium nitroprusside.

$\text{C}-\text{S}-\text{C}$, $\text{C}-\text{S}-\text{C}$, sulfites, creatinine, alkaloids, acetoacetic ester, etc. develop at the most only a slight orange color in 10 minutes. This color is not easily confused with the pink or purple of $\text{C}-\text{S}-\text{H}$.

No color is given with sulfones, sulfonic acids, urea, barbituric acid, taurine, thiophene, or isothioureas.

Among the compounds tested and found in the proper class are the following: (1) $\text{C}-\text{S}-\text{H}$: cysteine, glutathione, thioglycolic acid, thiosalicylic acid, butyl mercaptan, bismuth sodium thioglycolate. Thiophenol and thiocresol give fugitive purple-red colors only, the reagent being rapidly destroyed. (2) $\text{C}-\text{S}-\text{S}-\text{C}$: cystine, diglycylcystine, dialanylecystine, dithioglycolic acid, dibutyl disulfide, dibenzyl disulfide, diformidine disulfide. Negative tests were obtained with diparatolyl disulfide, diphenyl disulfide, and several other aromatic compounds in which S is directly linked to the ring. (3) $\text{C}=\text{S}$ and $\text{E}=\text{S}$: thiourea, allyl thiourea, tetramethyl thiourea, thiocarbanilide, thiobar bituric acid, thioacetic acid, potassium xanthate, sodium azido carbon disulfide, thioacetamide, potassium thiocyanate, sodium thiosulfate. Phenylisocyanate destroys the reagent in alcohol solution with a

transitory blue. Sodium diethyldithiocarbamate(C_2H_5) $\overset{\text{S}}{\parallel}\text{NC}-\text{SNa}$ destroys the reagent without coloration. Carbon disulfide gives only a faint green, probably because of lack of solubility but gives a blue after preliminary warming with sodium hydroxide solution.

Further work on the composition of the active reagent is in progress.

SUMMARY

1. A new color reaction has been found, apparently limited to compounds of divalent sulfur doubly linked to a single non-metallic element. The active reagent is produced by treatment of sodium nitroferriocyanide in sodium bicarbonate solution with hydroxylamine followed by bromine.

2. With use of the new reagent, a method is described to distinguish soluble organic compounds of the types $\text{C}-\text{S}-\text{H}$, $\text{C}-\text{S}-\text{S}-\text{C}$, and $\text{C}=\text{S}$ from other types and from one another.

3. The new reagent may be used for quantitative colorimetric estimation of thiosulfate, thiocyanate, thiourea, and other compounds of the $\text{C}=\text{S}$ type.

THE INFLUENCE OF GROWTH ON A NUMBER OF CONSTITUENTS OF THE WHITE RAT

BY ALFRED CHANUTIN

(From the Laboratory of Physiological Chemistry, University of Virginia, University)

(Received for publication, July 8, 1931)

While studying the effect of various experimental conditions on the chemistry of the whole white rat (gastrointestinal tract removed and designated as "eviscerated"), it became apparent that the concentration of several constituents changed rather markedly during the growth of the animal. Since the literature contained no detailed data concerning the chemical development of the rat, this investigation was carried out to study the creatine, nitrogen, ash, total solids, and ether-soluble materials in the growing and the adult rat.

Hunter (1) has presented the data for the ontogenesis of creatine in the skeletal muscle of a number of animals. He concluded that there is "a regular and uninterrupted increase in the concentration of muscle creatine, an increase commencing at a very early period of embryonic history, and ceasing only with the attainment, some time after birth, of the level appropriate to the adult." He further points out that the creatine concentration of the adult is reached while the animal is still immature.

The literature and data concerning the changes in the total solids, nitrogen, and ash concentrations in a number of mammals have been gathered by Moulton (2). He has pointed out a rapid increase in the percentage concentration of the constituents studied in the growing animal. There is no relationship between chemical and sexual maturity.

Buckner and Peter (3) have reported analyses of the ash content of the growing rat. In these experiments the weights given for the eviscerated animals were small for their age. This may explain the discrepancies between these results and those recorded below.

EXPERIMENTAL

The animals used in this work were raised in our laboratory and were maintained on a creatine-free stock diet. In studying the very young rats it was necessary to kill an entire litter, as a rule, in order to obtain sufficient tissue for analysis. Food and water were available up to the time of death. The methods used for the analysis of the "eviscerated" rats have been described in a previous paper (4). The young were allowed to remain with their mothers for about 30 days. No attempt was made to differentiate between male and female rats. Neither pregnant nor nursing animals were used for analysis.

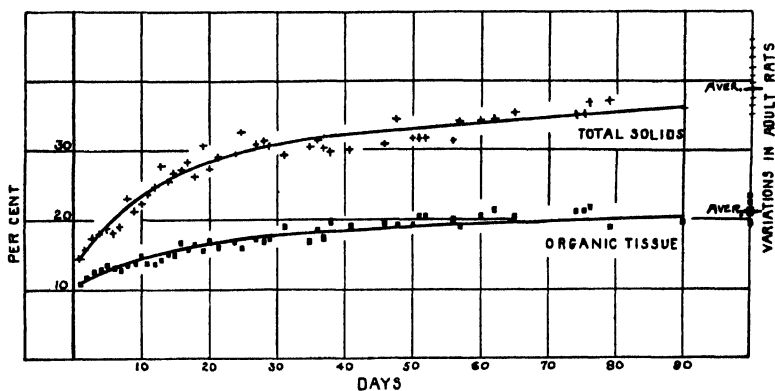


FIG. 1. Changes in the total solids and dry, fat- and ash-free tissue (designated as organic) of the growing rat. These and other curves have been placed by inspection.

A graphical analysis of the data reveals the following points. (1) The total solids and dried, fat- and ash-free tissue (designated as organic) increase progressively, especially during the 1st month of life. (2) The percentage concentration of nitrogen in moist tissue increases rapidly during the early days of life; on the other hand, the nitrogen concentration in the organic tissue remains extremely constant from birth to maturity. (3) There is a marked increase in the percentage concentration of ash in moist tissue up to the 20th day at which time "ash maturity" is reached; the increase in the concentration of ash in the dry and fat-free tissue is striking for this same period and is followed by a gradual fall to

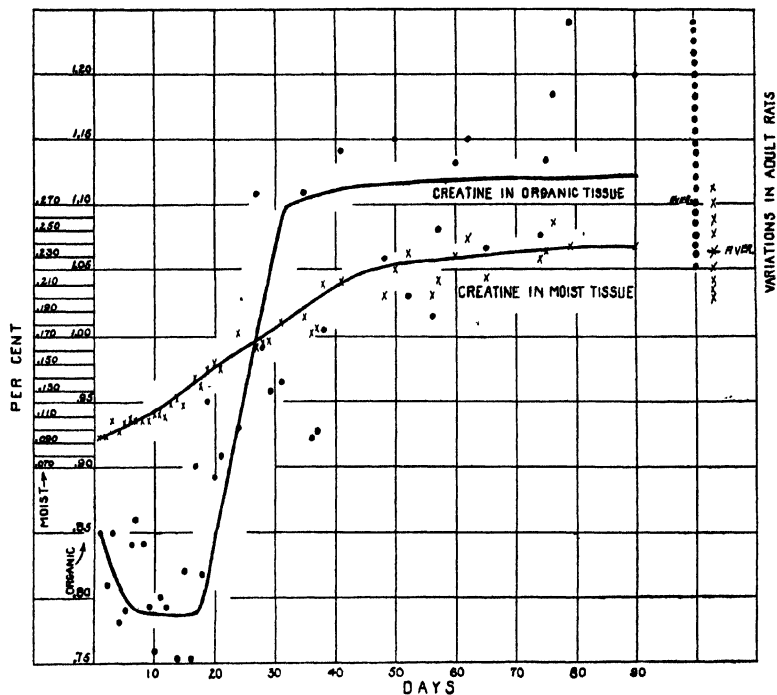


FIG. 2. Changes in the creatine concentration in the moist and organic tissue of the growing rat.

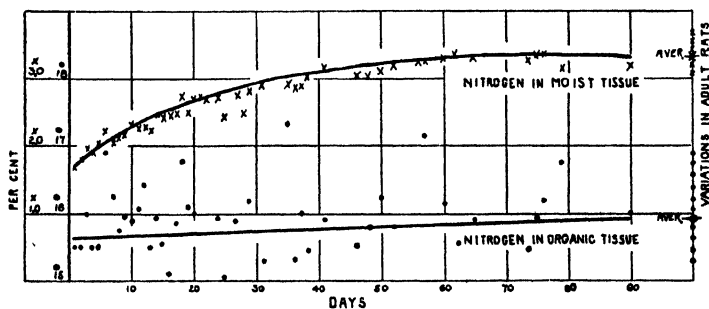


FIG. 3. Changes in the nitrogen concentration in the moist and organic tissue of the growing rat.

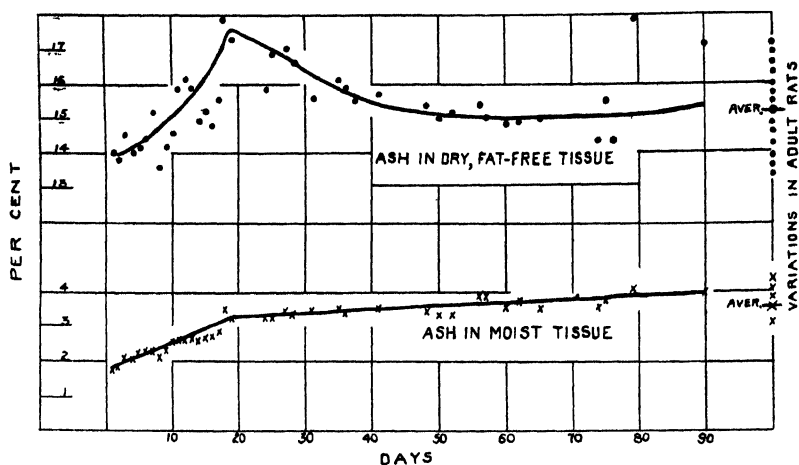


FIG. 4. Changes in the ash concentration in the moist and organic tissue of the growing rat.

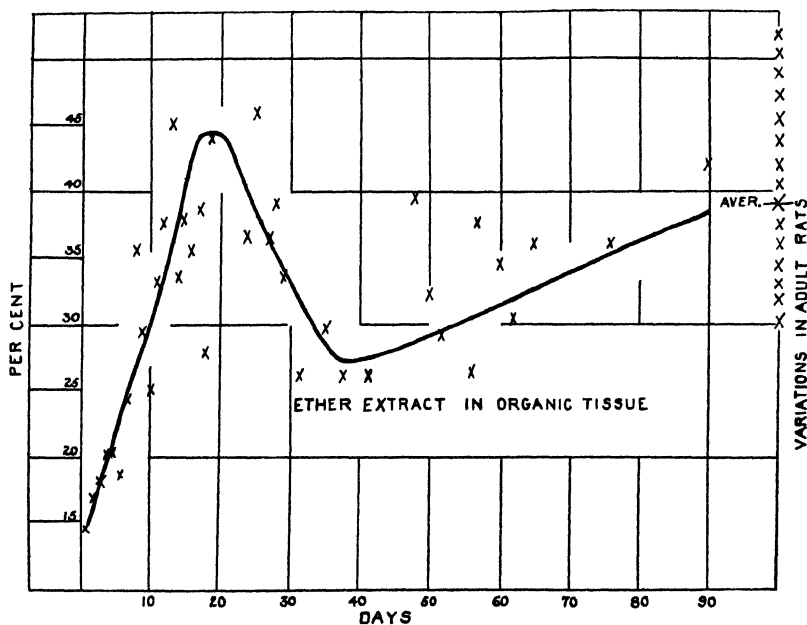


FIG. 5. Changes in the ether extract concentration in organic tissue of the growing rat.

the average ash concentration in the adult rat. (4) The percentage concentration of the ether extract in organic tissue increases markedly during the first 20 days of life; this rise is followed by a definite decrease. (5) The creatine concentration in moist tissue increases steadily in the growing rat and reaches the adult concentration at about the 40th day; the concentration in organic tissue presents a picture of an appreciable drop, shortly after birth, which is soon followed by a sudden and marked rise to "creatine maturity" at about the 30th to 40th day.

Each point on a graph (Figs. 1 to 5) represents at least three individual rats or litters. A minimum of five and a maximum of fourteen litters of the same age were analyzed to obtain the data for the first 16 days. In order to check the data at frequent intervals six to eight animals of the same age were sacrificed for analysis. Discrepancies in the weights of animals of the same age in different litters can be explained by the size of the litter and the sex of the animal. This is usually reflected in the fat analysis. An effort was made to obtain an equal distribution of sexes. The analyses given for adult animals were made on rats 150 or more days of age.

DISCUSSION

The critical period in the chemical growth of the rat occurs about the time of weaning (20 to 30 days). At this point the total solids, fat, ash, and creatine have either reached or closely approximated "chemical maturity." Mitchell and Carman (5) and Benedict and MacLeod (6) have demonstrated a high basal metabolism in rats aged 30 to 40 days. No data are available for younger animals. It is quite possible that rats at this age are undergoing a metabolic as well as a chemical "crisis."

The changes in the total solids noted in these studies are similar to the data recorded by Moulton (2), Hatai (7), and Sinclair (8). The initial concentration of 14.4 per cent is more than doubled on the 19th day to 30.4 per cent. The concentration of the total solids in the adult is reached about the 60th day. Much of the increase noted during the suckling period is due to the deposition of fat and to a lesser extent to the increase in ash.

The creatine concentration of the whole "eviscerated" animal increases gradually with the growth of the animal, a fact that has

been noted in muscle by other investigators. Creatine reaches the concentration for the adult between the 40th and 50th day. The data obtained for the organic tissue present an entirely different type of curve. The drop noted during the first few days of life is a significant one. This may be due to the loss of creatine stored during fetal life. However, there is no proof to substantiate these data. The increase noted at the 20th day is a very striking one. Although the creatine concentration is not consistently increased to the same degree, there appears to be a very definite trend towards an adult creatine concentration between

TABLE I
Changes in Various Components in the Growing Rat

Age	No. of rats	Weight of eviscerated rats	Fat	Ash	Total N	Total creatine
<i>days</i>		<i>gm</i>	<i>gm.</i>	<i>gm.</i>	<i>gm</i>	<i>gm</i>
1	13	4 7	0.08	0.08	0.08	0.004
9	9	10 3	0 55	0.23	0 22	0 011
14	14	14.3	1 07	0 38	0.35	0 017
19	7	25 3	2 98	0 82	0 69	0.037
27	6	31 0	3 00	1 10	0.85	0 051
31	8	42 5	2.93	1 48	1 23	0.078
41	5	66 6	4.43	2.46	2 09	0.141
50	7	91.0	8 20	3 07	2 83	0 200
57	6	85.3	9.60	3.34	2.75	0.180
62	6	114.4	10.50	4.25	3 78	0 280
75	4	125 0	12.20	4 82	4.18	0 297
90	4	126.2	17 50	5.13	4 22	0 297
Adult	39	224 3	30 0	8.57	7.56	0 525

the 30th and 40th day. The marked change in the organic creatine concentration within a comparatively short time is an extremely important fact in any study of the creatine metabolism of the young rat.

Hatai (7) has noted the increased fat content in the suckling rat with the subsequent decrease after weaning. He noted a much lower fat content in the mature animal than is recorded in these experiments. The increased metabolism and activity together with the change in diet at weaning must account for much of the decrease in fat content. A maximum increase in phospholipid fatty acids was noted on the 20th day by Sinclair (8).

The nitrogen concentration of the whole adult rat compares favorably with the results obtained by other investigators (Terroine (9)). Despite the changes associated with growth and metabolism, the organism maintains a very constant nitrogen concentration in organic tissue from the time of birth to adult age. An analysis of the data compiled by Moulton (2) would seem to indicate that this fact holds true for a large number of mammals.

It is well known that the deposition of salts in bone is greatest during the suckling period. Our data confirm this fact.

The actual changes in the growth of the various constituents studied are given in Table I. Representative groups have been chosen to present these data. These figures have been obtained by multiplying the weight of the "eviscerated" rat by the percentage concentration of the moist tissue. The amount of ash, total nitrogen, and total creatine is increased about 10 times during the first 19 days of life. Fat is being deposited at an extremely rapid rate as is evidenced by an increase of 35 times the fat content found at birth. The increases noted during the suckling period are the most pronounced during the life of the animal.

SUMMARY

The data presented show:

1. That the creatine concentration of the rat reaches chemical maturity between the 30th and 40th day.
2. Both fat and ash concentrations reach a maximum at the 20th day after which there is a gradual but marked decrease.
3. The nitrogen concentration of the organic tissue remains constant throughout the life of the rat.
4. The greatest increase in the constituents studied occurs during the suckling stage of life.

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HEMOGLOBIN PRODUCTION

III. THE RELIEF OF ANEMIA, DUE TO MILK DIET, BY FEEDING AMINO ACIDS AND RELATED COMPOUNDS*

BY DAVID L. DRABKIN AND H. K. MILLER

(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia)

(Received for publication, June 25, 1931)

Additional experiments upon the relief of milk anemia in rats by feeding amino acids and related compounds (1) have been carried out. These experiments will be presented, as they were performed, in three groups: Group A, the testing of the effectiveness in hemoglobin regeneration of supplementing milk with such substances as leucine, cystine, glycine, α -amino valeric acid, glutaric acid, succinic acid, and succinimide; Group B, the study of the prevention of milk anemia; and Group C, the determination of the quantity of glutamic acid and iron needed to produce appreciable increases in hemoglobin in anemic rats. The latter experiments were essential, since in the earlier work (1) the quantities of metallic and organic supplements were chosen empirically.

Methods

The albino rats used in this investigation were kept in the same cages during the whole course of the experiment, one or two animals per cage. From weaning, in Groups A and C, the diet was raw cow's milk. After the development of severe anemia, the rats were fed milk which was modified so as to furnish 0.18 mg. of iron per animal per day as well as a definite quantity of organic supplement. The milk and the supplements were fed as quantitatively as such experiments permit. A record of daily food

* An abstract of this paper was presented to the American Society of Biological Chemists at the Montreal Meeting, and has appeared in the Proceedings of the Society (Drabkin, D. L., and Miller, H. K., *J. Biol. Chem.*, **92**, p. lxi (1931)).

A part of the expense of this work has been met by a fund donated by Merck and Company, Incorporated.

consumption was kept. Since 70 mg. of glutamic acid were found empirically, in the earlier work, to be an effective supplement, the quantities of organic substances fed in the present experiments were 1, 2, or 4 equivalents on the basis of molecular weight to the above level of glutamic acid.

In those experiments (Group B) in which the prevention of milk anemia was studied, the milk was supplemented at the start with either iron alone (so as to furnish 0.18 mg. per rat per day), with iron plus alanine, or with iron plus sodium hydrogen glutamate. To test whether boiled milk was possibly less effective in producing anemia than raw milk, one set of animals in this group was fed boiled milk plus iron.

In the study of the quantity of glutamic acid and iron needed for the relief of milk anemia (Group C) the amino acid was fed in varying quantities as the monosodium salt. The free acid and its salt were equally effective (1) in hemoglobin regeneration, but the sodium hydrogen glutamate is far more soluble. In feeding 70 mg. of glutamic acid 7.5 cc. of solution had to be added to the milk, whereas an equivalent quantity of the sodium salt could be administered in 2 cc. of water. From the standpoint of comparable fluid intakes in the various experiments the smaller quantity was considered preferable.

For the methods of hemoglobin, iron, and copper analysis and additional details as to procedure the reader is referred to the previous publications (1, 2).

EXPERIMENTAL

Group A—In these experiments, the results of which are shown in Chart I, the curative value in milk anemia of various organic substances was tested. In terms of molecular equivalents of 70 mg. of glutamic acid, 1 equivalent of leucine, 1 equivalent of cystine, 4 equivalents of glycine, 2 equivalents of *dl*- α -amino valeric acid, and 2 equivalents of glutaric acid were ineffective in the relief of milk anemia. With the first two amino acids (leucine and cystine) there was an apparent arrest of the progressive anemia, but no indication of increase in hemoglobin. Even though milk was supplemented with glycine before the rats were highly anemic and 4 times the effective glutamic acid level was fed, the progressive course of the anemia was not checked. In

contrast to the above substances, 2 equivalents of succinic acid and 4 equivalents of succinimide were somewhat effective in the

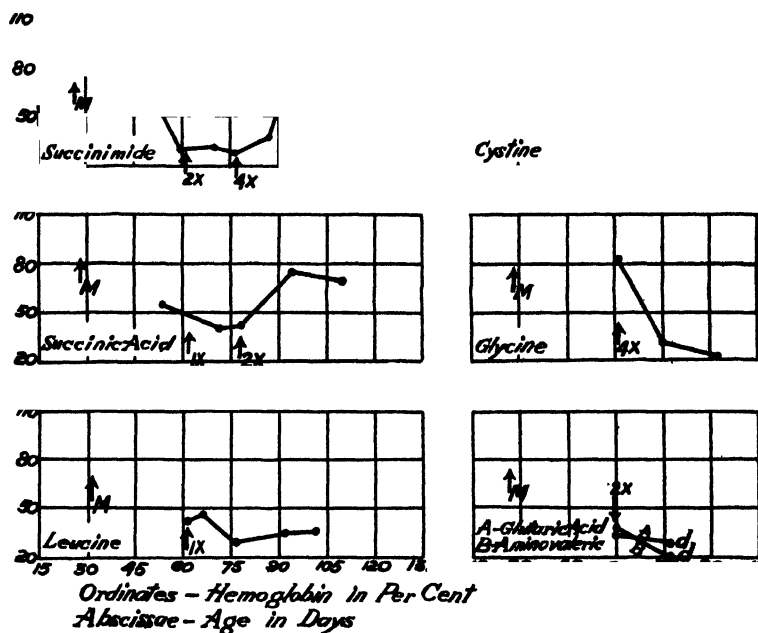


CHART I. Arrows M indicate the points at which rats were placed upon whole milk; 1X, 2X, and 4X indicate the points at which milk was supplemented with 0.18 mg. of iron per rat per day and with 1, 2, or 4 equivalents (on the basis of molecular weight to 70 mg. of glutamic acid) of various organic substances; d indicates death of animal. Four rats were used in each of the experiments, only one typical result being shown. The supplements were added in water solution to the daily ration of milk. Succinimide was added in amounts of 1, 2, and 4 cc., containing 47.2 mg. per cc.; succinic acid in amounts of 2 and 4 cc., 56.2 mg. per 2 cc.; l-leucine in amounts of 3 cc., containing 62.4 mg.; glycine in amounts of 2 cc., containing 144 mg.; glutaric acid in 1 cc. quantities, containing 126 mg.; dl- α -amino valeric acid in 2 and 4 cc. quantities, 112 mg. per 2 cc. To insure solubility, l-cystine was added in 2 cc. amounts, containing 114 mg. of the amino acid and 38 mg. of NaOH.

regeneration of hemoglobin, though smaller quantities of these compounds showed no effect.

The various supplements, except the cystine which was made

in our laboratory and the succinimide which was recrystallized by us, were preparations purchased in the market. The nitrogen content and optical activities of the various products were found to be in satisfactory agreement with the theoretical.

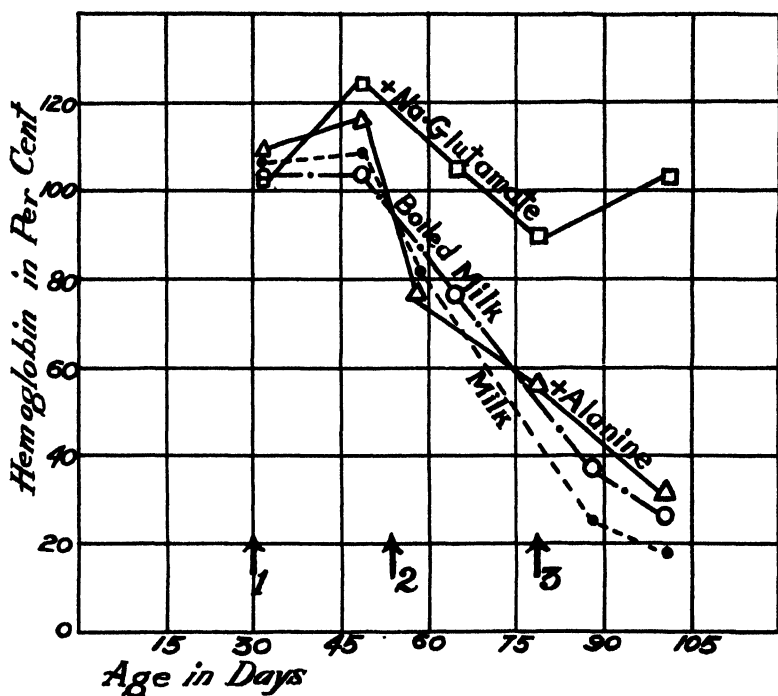


CHART II. Arrow 1 indicates the point at which rats were placed either upon raw whole milk, boiled milk, raw milk supplemented with 80 mg. of sodium hydrogen glutamate corresponding to 70 mg. of glutamic acid per rat per day, or raw milk supplemented with 84 mg. (2 equivalents) of alanine per rat per day. At this same point (Arrow 1) the ration of all the rats was also supplemented so as to furnish 0.18 mg. of iron per animal per day. In each group of experiments two rats were used. Arrow 2 marks the point where the iron supplement was discontinued in all the experiments; Arrow 3, the point where iron salt was again fed to all rats at a level of 0.09 mg. per rat per day.

The analyses for iron and copper are of interest mainly in the case of the effective substances. 1 gm. of succinic acid was ash-free (*i.e.*, ash less than 0.01 per cent). A 0.5 gm. sample con-

tained 0.004 mg. of iron and had too little copper to be detected by the methods of analysis. The ash content of the succinimide was 0.02 per cent. 0.005 mg. of iron was found in a 0.5 gm. sample. A sample of this magnitude had too little copper for analysis. In the case of the ineffective supplements it is of interest to note that, in respect to iron, cystine is the most highly contaminated amino acid thus far used by the writers. The ash content of our preparation was 0.26 per cent, while the iron content was 0.01 mg. per 0.5 gm. In this instance also, however, a 0.5 gm. sample had too little copper to be determined by the methods used by us.

Group B—Chart II indicates the following: (1) The feeding of boiled instead of raw milk had no apparent prophylactic value as far as the development of anemia was concerned. (2) The onset of anemia was effectively retarded by the administration of an iron salt at a level (0.18 mg. of iron per rat per day) which was ineffective in curing the anemia once produced (1). (3) Rats fed milk supplemented with iron and sodium hydrogen glutamate from weaning had higher hemoglobin levels than those on milk plus iron alone. Upon the withdrawal of the inorganic supplement, there was definite evidence that the animals receiving the glutamic acid salt were more resistant to the development of anemia than those fed milk supplemented by iron alone or by iron and alanine.

Group C—The results obtained in the experiments upon the quantity of glutamic acid and iron needed for the relief of milk anemia are shown in Chart III. The results are expressed in terms of glutamic acid, but, as has been stated, the amino acid was fed in equivalent quantities of its monosodium salt. A definite increase in hemoglobin was observed when 35 cc. of milk, supplemented with 0.18 mg. of iron and 35 mg. of glutamic acid, were fed to anemic rats. At this level of iron feeding, some regeneration of hemoglobin was also evident with 17.5 mg. of glutamic acid, while 8.8 mg. of the amino acid were ineffective. It is to be noted that even with this very small quantity of glutamic acid a slight increase in hemoglobin was observed when it was added to the milk before the animal had become severely anemic.

When the milk was supplemented so as to furnish 70 mg. of glutamic acid per rat per day there was a definite rise in hemoglobin with only 0.09 mg. of iron. With the same quantity of glu-

tamic acid there was a slight temporary stimulation with as little as 0.05 mg. of iron. Glutamic acid administration without iron was ineffective.

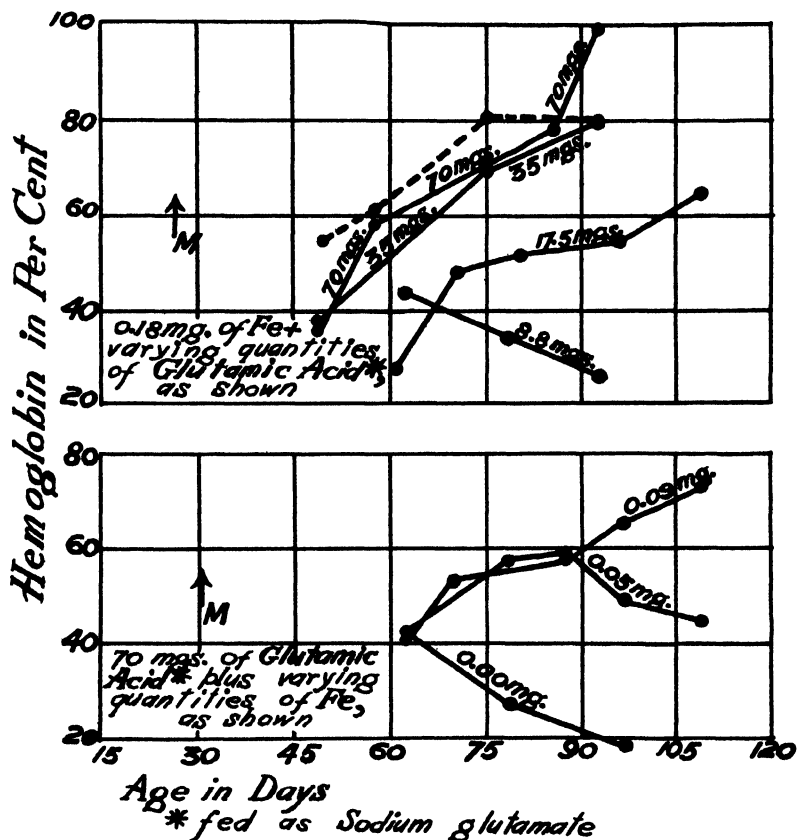


CHART III. Arrows M indicate the points at which rats were placed upon whole milk. Each curve represents two rats. The supplements, indicated in the chart, were added to the milk at the height of the anemia. The broken line represents a second group of two rats, which were fed 8.8 mg. of glutamic acid in the form of the sodium salt. In this case the supplement was started at a higher level of hemoglobin.

DISCUSSION

The finding that both succinic acid and succinimide, when fed in appropriate quantities, stimulate hemoglobin production is of

interest. These substances were used because aspartic acid was found somewhat effective (1). It is to be noted, however, that hemoglobin production with these supplements, although better than with aspartic acid, was less pronounced than with glutamic acid. If 17.5 mg. of glutamic acid are used for comparison and relative molecular weights are considered, no hemoglobin increase was observed until the milk was supplemented with 8 times this quantity of succinic acid and 16 times that of succinimide. At the present time, however, this comparison may mean little since we are dealing with substances of different length of carbon chain, and we know nothing concerning their relative absorption or utilization.

Glutaric acid is related structurally to glutamic acid in the same way that succinic acid is to aspartic acid. For this reason its value in hemoglobin production was tested. This supplement proved to be presumably as toxic for the rat as Rose (3) has found it to be for the rabbit.¹ The consumption of diet was poor and whether or not the negative results with glutaric acid were due to its toxicity cannot be said.

From the standpoint of its structural relationship to arginine, one might have hazarded a guess that *dl*- α -amino valeric acid would be found effective in the relief of milk anemia. Such was not the case, however. In this instance also the result may or may not be related to the toxicity of the material.¹

The study of the value of boiled milk in the prevention of anemia is another example of the failure of a hypothesis to materialize. The value of "dried" milk as a prophylactic agent against milk

¹ As in the previous work Dr. George M. Robson of the Department of Pathology is making a careful, histological study of the essential organs of the rats used in our experiments. This phase of the work will be reported when a sufficient accumulation of data warrants it. It may be said here, however, that the rats receiving glutaric acid showed severe liver and kidney injury. Some liver injury was also observed in the rats fed α -amino valeric acid. The tissues of the rats receiving the other supplements were essentially normal. With reference to the evidence of erythropoiesis in the liver and spleen which had been found in the case of arginine feeding (1), it is of interest to note that the spleen of the rats fed leucine showed extreme erythropoiesis, although the hemoglobin of these animals did not increase. The significance of these morphological findings may become apparent after further studies which are in progress.

anemia has been reported (4). The idea that boiled milk might be better utilized by the rat than raw milk and thus serve to explain in part the mechanism of anemia production received no support from our experiment. In this group of experiments (Group B) by far the most striking finding was the apparent increase in resistance to anemia of rats receiving sodium hydrogen glutamate. How this decrease in susceptibility to the production of anemia is accomplished remains to be elucidated.

In the previous paper (1), it was calculated that, in terms of the glutamic acid content of milk (5), the addition of 70 mg. of glutamic acid to the daily milk ration increased the amount of this amino acid by approximately 40 per cent. If it be assumed that hydroxyglutamic acid acts similarly to glutamic acid, the presence of this amino acid in milk (6) must also be considered in such a calculation. When this is done, the addition of 70 mg. of glutamic acid only increases by about 28 per cent the total glutamic acids. Since, in the above experiments, as little as 17.5 mg. of glutamic acid were observed to stimulate hemoglobin production, it becomes evident that an increase in glutamic acid of only 7 per cent was nevertheless effective. The writers are aware that such a result appears rather paradoxical and demands experimental explanation.

Simultaneously with the publication of Paper II of this series, further evidence of the value of tryptophane in hemoglobin production was presented by two groups of investigators (7). At the same time Whipple and his fellow workers (8) described very interesting experiments, which presumably have permitted the postulation that the pyrrole nucleus of hemoglobin can be synthesized by the animal body. The recent important paper of Dakin and West (9) suggests that the effectiveness of liver extract in pernicious anemia is bound up with the presence in the extract of building stones for the synthesis of the pyrrole portion of hemoglobin. There seems to be no doubt of the therapeutic value of the pyrrole derivatives thus far isolated, but the quantities of materials which are used in pernicious anemia appear too small in comparison with the total quantity of hemoglobin regenerated to be accounted for solely on the basis of *pyrrolegenesis*. The latter term was introduced by us (1) to describe briefly the production or origin of pyrrole groups in the metabolism of *heme*. *Pyrrolegenesis* was offered as one of the working hypotheses to explain the effec-

tiveness of various amino acids and related substances in the relief of milk anemia in rats. The quantities of materials used in our work, although much larger proportionately than those employed by Dakin and West, are nevertheless far smaller than the amount of similar substances already present in the milk upon which rats become anemic. It is also well known that therapy with sufficient amounts of the salts of heavy metals is in itself adequate in curing the anemic rats. Thus, at least in this type of anemia, much further work is necessary to establish the validity of the *pyrrolegenesis* hypothesis.²

SUMMARY

Experiments have been performed which indicate that, while succinic acid and succinimide are somewhat effective in the relief of milk anemia, leucine, cystine, glycine, α -amino valeric acid, and glutaric acid are ineffective.

Rats have been found to become anemic as readily upon boiled as upon raw milk. The onset of anemia has been retarded by the administration of an iron salt at a low level which was ineffective in curing the anemia once produced. Rats receiving milk supplemented with sodium glutamate appeared definitely more resistant to anemia, even in the absence of iron administration, than rats which were fed iron alone or iron and alanine.

The results of a third group of experiments in which the quantity of glutamic acid and iron needed for the relief of milk anemia has been studied have been reported and discussed.

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² Dr. Thomas Fitz Hugh of the Department of Medicine is conducting a study of the therapeutic value of glutamic acid in patients with anemia. Thus far it has proved exceedingly difficult to obtain cases of secondary anemia suitable for a critical study. In two well controlled cases of pernicious anemia, however, glutamic acid was ineffective, whereas, liver therapy produced the expected response.

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THE RÔLE OF COPPER IN HEMOGLOBIN REGENERATION AND IN REPRODUCTION

BY H. L. KEIL AND VICTOR E. NELSON

(From the Laboratories of Physiological Chemistry, Iowa State College, Ames)

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The relation of copper to hemoglobin formation has attracted the attention of a number of investigators during the past few years. Hart *et al.* (1) were the first to point out that copper plays a specific rôle in hemoglobin building, and that milk fortified with pure iron salts does not constitute an adequate diet for regeneration of the respiratory pigment. Evvard, Nelson, and Sewell (2) showed that rats and swine make better gains and exhibit a higher food utilization per unit of weight increase when small amounts of copper sulfate are incorporated in the ration. In the ashing of the rats fed copper sulfate, it was observed that the greater part of the stored copper was confined to the liver. The authors stated, "It is possible that the medicinal and nutritive value of liver and its proper functioning may be somehow related to this element." McHargue, Healy, and Hill (3) practically simultaneously with Hart and his coworkers observed that copper stimulated hemoglobin regeneration of rats on an anemia-producing diet.

Further evidence for or against the necessity of copper in the formation of hemoglobin has been presented by Lewis, Weichselbaum, and McGhee (4), Krauss (5), Elden, Sperry, Robscheit-Robbins, and Whipple (6), Drabkin and Waggoner (7), Myers and Beard (8), Mitchell and coworkers (9), Keil and Nelson (10), Titus, Cave, and Hughes (11), and Underhill, Orten, and Lewis (12). Only a superficial examination of the literature is necessary to reveal that there is considerable divergence of opinion regarding the rôle and necessity of copper in hemoglobin building. Drabkin and Waggoner (7) state:

"If the milk were copper-free, the problem would be far simpler. One could then definitely assume that copper is a specific supplement to iron in

hemoglobin building. Since, however, anemia-producing milk contains copper and a cure of the anemia has been accomplished by synthetic diets which are lower in copper, in this type of experiment the specificity of copper, or even its necessity, becomes highly questionable."

Myers and Beard (8) found that iron alone was sufficient for regeneration of hemoglobin. They found, furthermore, that other elements besides copper can increase the speed of regeneration. Particularly did they observe that manganese, nickel, germanium, and arsenic have this effect. Mitchell and Miller (9) believe that a group of elements is active in hemoglobin building rather than copper alone. Titus, Cave, and Hughes (11) had already stated that manganese plays a part in the synthesis of hemoglobin. Keil and Nelson (10) found that regeneration of hemoglobin occurred on pure FeCl_3 plus milk, but that the speed of regeneration was greatly increased by the addition of a small amount of copper sulfate.

EXPERIMENTAL

The hemoglobin content of 6 months old rats on our growing ration varies from 14 to 18 per cent. When these same animals are placed on a whole milk diet on glass screens in glass cages or in galvanized wire cages, with galvanized screen bottoms, the hemoglobin of the blood falls to a level of 2 to 8 per cent in 5 to 7 weeks time. The ferric chloride used in our experiments was prepared either from Baker's analyzed standard iron wire or electrolytic iron. The iron was dissolved in concentrated redistilled HCl to which was added a small amount of c.p. HNO_3 ; the solution was evaporated to small volume and H_2S was bubbled through the dilute solution in order to precipitate any copper that might be present. The solution was then filtered and made ammoniacal and the ferric hydroxide filtered off and washed repeatedly with copper-free water. The ferric hydroxide was then dissolved in redistilled c.p. HCl and the crystals of ferric chloride were filtered off after standing. Since the ferric chloride contains water of crystallization which may vary considerably under different conditions, the ferric chloride was dissolved in copper-free water and the amount of iron was determined colorimetrically by the KCNS method. This insured a more quantitative administration of iron. The FeCl_3 , stock solution of FeCl_3 , and solution of FeCl_3 ,

as fed to the animals were all examined by a Hilger quartz prism spectrograph and shown to be copper-free. Carbon electrodes were used in the spectrographic examination. Hemoglobin was determined by the Newcomer method. The animals were bled by the tail.

The following elements were studied: vanadium, titanium, manganese, copper, nickel, arsenic, germanium, zinc, chromium, cobalt, tin, and mercury. All of the elements were fed at a daily level of 0.05 mg. except manganese, which was given at a 0.1 mg. level; and germanium, which was incorporated in the ration at a level of 0.09 mg. per day. As a source of vanadium, V_2O_5 was used. The oxide was warmed with concentrated HCl and subsequently the solution was diluted with water to the required volume. c.p. TiO_2 was used as a source of titanium. The TiO_2 was dissolved in hot concentrated sulfuric acid and made up to volume. c.p. As_2O_3 was resublimed and dissolved in water to the required concentration. Zinc chloride, mercurous nitrate, and chromium acetate were used as sources of zinc, mercury, and chromium. Metallic germanium was dissolved in aqua regia, as little of the latter as possible being employed, and the mixture was slightly warmed. $SnSO_4$ was used as a source of tin. It formed a cloudy suspension in water so that the mixture was well shaken before using. Copper nitrate was prepared by dissolving electrolytic sheet copper in HNO_3 and evaporating to dryness, subsequently dissolving in water. c.p. manganese chloride, c.p. cobalt chloride, and c.p. nickel sulfate were employed as sources of manganese, cobalt, and nickel. The latter three salts were placed in solution in 0.25 N HCl of known purity and H_2S was bubbled through the solution for 4 hours. After filtration the salts were crystallized. All of the salts were copper-free according to spectrographic examination.

The curves show the results obtained. It is self-evident that the iron and milk did not cause regeneration of the hemoglobin. Copper is the only element of all those tested which has the capacity to stimulate hemoglobin building. Since copper exists as a monovalent and divalent element it was thought that other elements, especially V, which occurs in several states of valency, might show the same properties in hemoglobin regeneration as copper, but such was not the case. Our data do not harmonize

with the data of Titus, Cave, and Hughes, Beard and Myers, or Drabkin and Waggoner; and they convincingly demonstrate that notwithstanding the contrary results of others copper possesses unique powers in hemoglobin synthesis as demonstrated by Waddell, Steenbock, and Hart (13). We ascribe our previous results which conflict with our most recent data to the fact that the milk used in previous experiments contained sufficient copper for regeneration. Our first experiments were performed with groups of rats in a cage and with market milk and pure iron as ferric chloride. Results were always positive on hemoglobin building. When the rats were placed in individual compartments the same results were not obtained. However, the results were not uniform. No doubt two factors played a part in the discrepancy of these results, namely coprophagy and the copper content of the milk. This milk was obtained from the college creamery and, although all vessels were well tinned, nevertheless, the copper content of the milk was sufficient for regeneration of the hemoglobin. The copper content of the market milk, as determined by the sodium diethyldithiocarbamate method, gave results between 0.35 and 0.44 mg. per liter. These values are within the lower range of certain data published by Supplee and Bellis (14). The results tabulated in this paper (Charts I and II) are the outcome of experiments continually in progress for the past year and a half. Each curve is a composite for five to ten animals. When market milk of the above composition was used addition of iron as FeCl_3 caused regeneration so that in a few weeks time the normal value of hemoglobin was attained. The milk employed in the experiments recorded in this paper was collected directly in glass containers from two pure bred Holstein cows. It contained 0.24 mg. of copper per liter by the carbamate method of analysis. On this latter milk together with pure iron in the form of FeCl_3 animals became anemic, so that the hemoglobin fell to 2 to 5 per cent from a level of 15 to 18 per cent. We are convinced that Hart, Steenbock, and coworkers are correct in their postulation regarding copper in hematopoiesis. Neither the market milk nor the milk collected in glass was analyzed daily to determine the copper content, but from the periodical analyses made, it appears that the latter milk was lower in copper content than the former. At

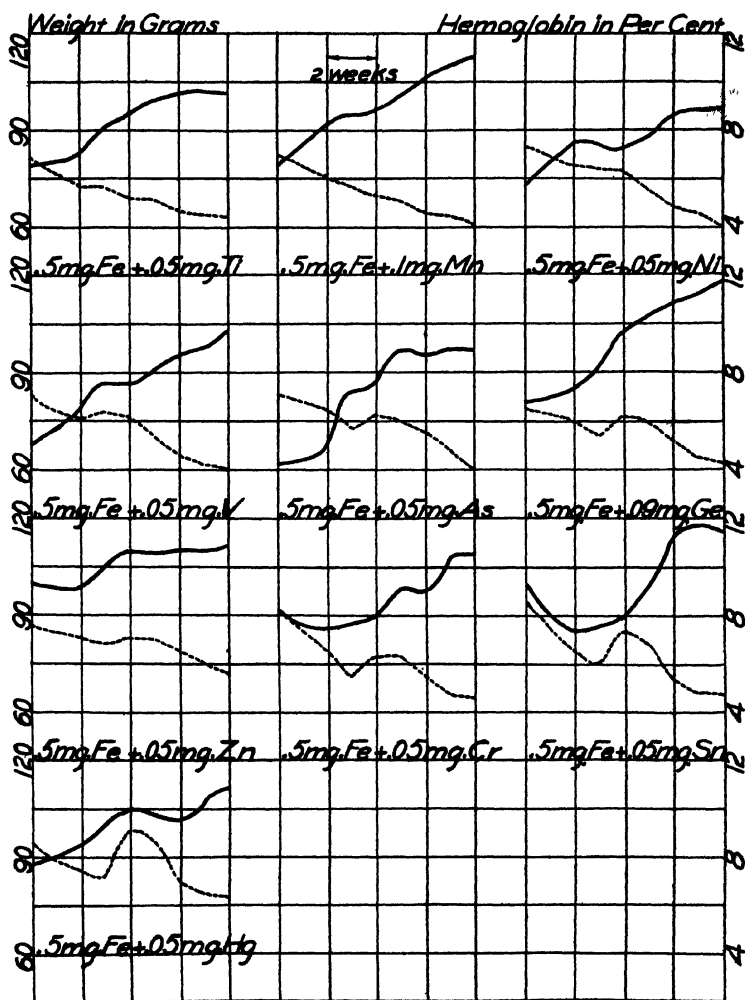


CHART I. Results of adding iron as ferric chloride and salts of various metals to the basal diet of cow's whole milk. Germanium, zinc, chromium, tin, and mercury salts were added at the end of 3 weeks. During the first 3 weeks the animals were on whole milk plus iron as ferric chloride. The remaining animals were placed on whole milk for 1 month until anemia developed (not shown on curve), and then the respective elements were added to the milk and iron. The curves are composite. The solid lines show growth and the broken lines indicate hemoglobin.

least we are convinced that with milk collected in glass together with pure iron hemoglobin regeneration is impossible.

We have also been interested in the rôle of copper in reproduction. Daniels and Hutton (15) showed that reproduction on milk and iron was greatly improved by the addition of soy bean, ash of soy bean, or a mixture of mineral constituents containing manganese, fluorine, aluminum, and sodium silicate.

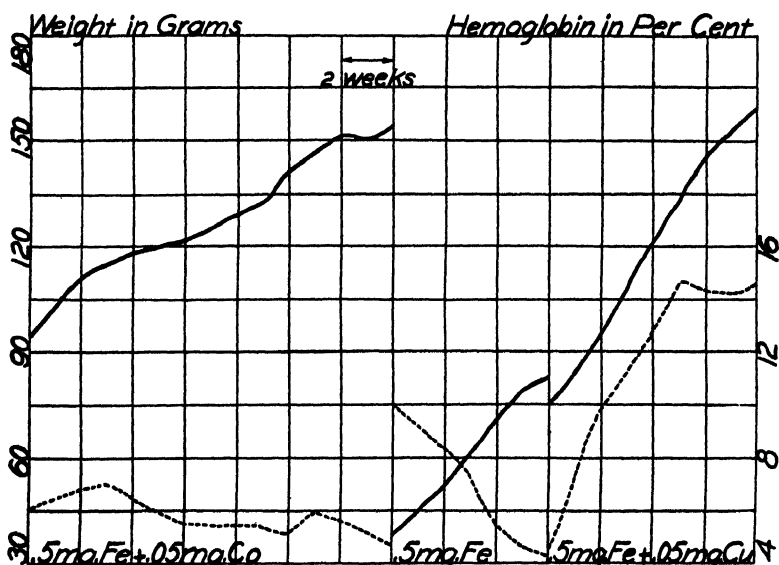


CHART II. Showing the effect of cobalt, iron, and copper on regeneration of hemoglobin. The group on iron received whole milk plus iron as ferric chloride as soon as weaned. The animals on cobalt and copper were first made anemic on whole milk (not shown on curve), and then were given iron as ferric chloride together with the salt of the metals. The solid lines show growth and the broken lines hemoglobin.

A group of rats (nine females and four males) were placed on a diet of market milk for 6 months. The animals weighed between 50 and 60 gm. when placed on the milk diet. After 6 months five females and two males received in addition to the milk 0.5 mg. of iron as FeCl_3 daily. The remaining four females and two males received 0.05 mg. of copper as copper sulfate daily in addition to the milk and iron. Only one of the females on the milk and FeCl_3

diet reproduced in 5 months. This female had eight young. On the milk, iron, and copper ration one female had four litters of twenty-three young, a second female had five litters of thirty-one young, a third female had five litters consisting of thirty-three young, while the fourth female had five litters of thirty-seven young in 5 months time. An attempt was made to raise four of the litters. Three of the litters were consumed by the mothers. One female reared seven young from a litter of ten with an average weaning weight of 51 gm. in 30 days. These data are in contrast with the results of Krauss (16) who states, "In spite of the fact that we have kept males and females together for long periods on such a supplemented (copper and iron) milk diet, no offspring have ever been produced." We are unable at present to account for the conflicting results. It may lie in the difference in copper content of the rations or in the difference in reserve stores of copper in the animals employed. Nor are we able to explain our results in terms of the data obtained by Daniels and Hutton (15). It is probable that the mineral components used by the latter investigators may play a part in reproduction and especially lactation. Time has not permitted us to determine the factors involved for optimum reproduction or lactation, nor have we determined the optimum concentration of iron and copper for reproduction and milk secretion. This we expect to do. The fact remains that animals have reproduced on milk, iron, and copper and we hope subsequently to unravel the reason for conflicting view-points. The hemoglobin values of the blood of the female animals on milk and iron expressed in per cent were as follows: 12.0, 7.6, 10.0, 11.9, and 11.2. The hemoglobin of the blood of the female animals on milk, iron, and copper expressed in per cent was as follows: 14.6, 14.1, 13.6, 15.0, 15.8, and 13.5. These determinations were made on the animals when 11 months of age. The animals are now 12½ months old.

Nutritional anemia produced by whole milk causes a change in color of the coat of the animal. This change occurs particularly in the dark coated rats. Black or black hooded animals replace the black color with a silvery gray that is very striking. The gray coated animals change to a buff or silvery gray with a yellowish tint. The changes brought about in the color of the fur through lack of iron are very noticeable in about 8 weeks. The

addition of pure iron as ferric chloride does not restore the color; in fact, animals on whole milk plus pure iron as ferric chloride also develop this change in the color of the fur. The addition of 0.05 mg. of copper per day to the whole milk and iron as ferric chloride restores the original coat in about 2 months time.

SUMMARY

1. Nutritional anemia is readily produced in rats on a whole milk diet.

2. Pure iron as ferric chloride when added to milk collected in glass does not cause regeneration of hemoglobin.

3. Salts of vanadium, titanium, manganese, nickel, arsenic, germanium, zinc, chromium, cobalt, tin, and mercury also failed to stimulate regeneration of hemoglobin when added to milk, collected in glass, and supplemented with pure iron as ferric chloride.

4. Copper was the only element of those tested which had a positive effect on hemoglobin building.

5. Reproduction was obtained on milk and iron as ferric chloride by the sole addition of copper sulfate.

6. Nutritional anemia produces a distinct change in color of the dark coated rats.

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THE UNSAPONIFIABLE LIPIDS OF LETTUCE

II. FRACTIONATION*

By H. S. OLCOTT AND H. A. MATTILL

(From the Biochemical Laboratory, State University of Iowa, Iowa City)

(Received for publication, June 15, 1931)

The chemistry of green leaves has attracted increasing interest during the past few decades. Plant physiologist and organic chemist have sought a better understanding of the complex substances and processes concerned in the functions of this most active tissue of the plant, and students of animal nutrition, beginning with the pioneer work of Osborne and Mendel, of McCollum, and Steenbock, and their coworkers, have reaffirmed the intimate dependence of animal life upon the synthetic products of plant life. This is particularly true of the fat-soluble vitamins A, D, and E, whose common origin is ultimately traceable to the unsaponifiable constituents of the lipids of vegetable organisms.

Our knowledge of the many and varied lipid constituents of leaves is very fragmentary; not only do species differ mysteriously in the synthetic capacities with which their cells are endowed, but some of the products are present in amounts so small as to be quite insignificant in comparison with their importance. Furthermore only the recent advent of new methods, notably those of microorganic and absorption spectrum analysis have made it possible to differentiate between them. It is not surprising, therefore, that few systematic studies of plant lipids have thus far been made. Among the purely chemical studies are those of Channon and Chibnall (1) on cabbage and those of Heyl, Wise, and Speer (6) and Clenshaw and Smedley-MacLean (2) on spinach. Physiological studies are almost countless, dealing mainly with the distribution of the fat-soluble vitamins, the relative amounts found

* The data of this and the following paper were reported in part before the Montreal meeting of the American Society of Biological Chemists (Olcovich, H. S., and Mattill, H. A., *J. Biol. Chem.*, **92**, p. xxxi (1931)).

in different plants at different stages of growth, and their stability and permanence under the natural and artificial conditions to which they may be subjected.

Lettuce possesses interesting nutritional properties. Its high content of vitamin A has been established by many investigators; vitamin D was early produced in lettuce by ultra-violet radiation and when vitamin E was first recognized lettuce was found to contain it in higher concentration than any other substances tested. Recently (8) watercress has been found to surpass it. Furthermore, lettuce contains a growth-promoting factor (4, 7) which has thus far not been identified with any of the known accessories. A detailed investigation of the unsaponifiable lipids of lettuce was therefore undertaken in an attempt to segregate the physiologically active materials, and to study the accompanying compounds.

Drying, Extraction, Saponification

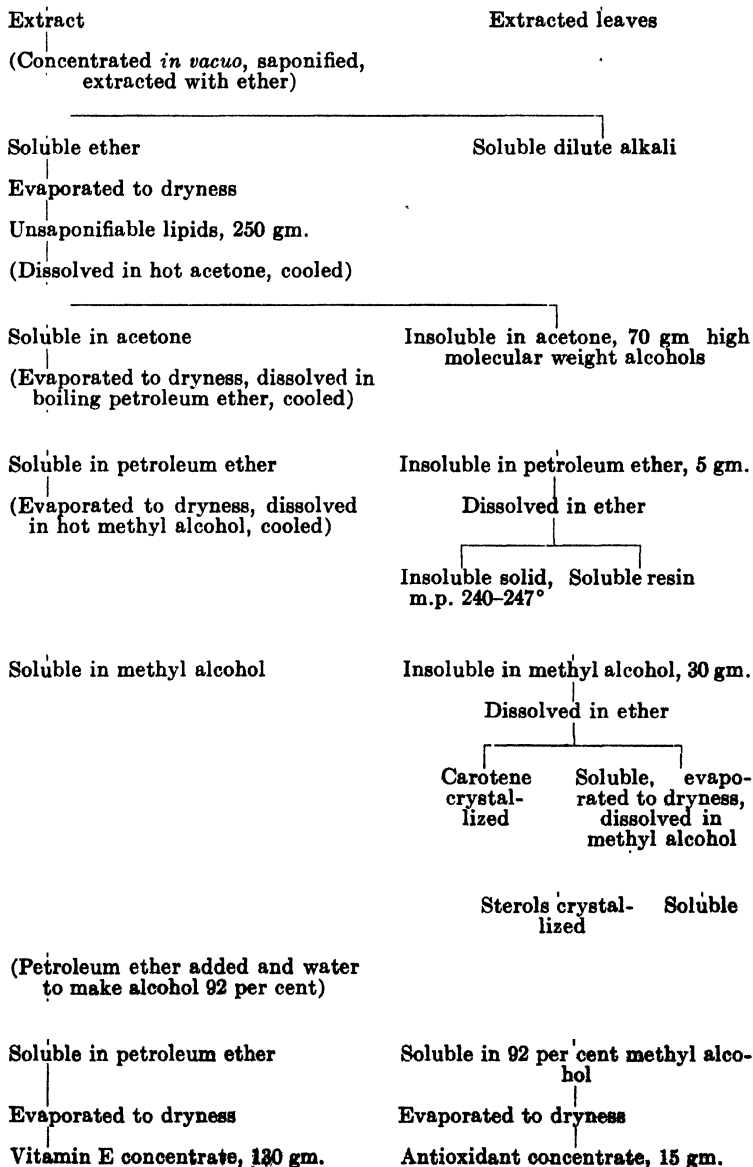
Fresh lettuce leaves, freed from their fleshy stems, were completely dried in a steam-heated vacuum oven in 4 to 5 hours. The dried leaves were powdered, and extracted with hot alcohol in continuous extractors for 20 hours. The alcoholic extracts were evaporated *in vacuo* to a thick liquid, and stick KOH was added with shaking (100 gm. for the extract of 2500 gm. of dried leaves). The mixture was allowed to stand overnight at 37°, the excess alcohol was evaporated *in vacuo*, and the saponified lipids were transferred to separatory funnels, diluted generously with water, and extracted nine to twelve times with peroxide-free ether.¹ The ether extracts were combined, washed, dried with anhydrous sodium sulfate, and evaporated to dryness *in vacuo*. The resulting amorphous, brown mixture constituted the unsaponifiable lipid fraction. Oxidation was reduced to a minimum by replacing the air in all procedures with an inert gas.

Fractionation

The scheme of fractionation is outlined in Diagram 1. The method is essentially that used by Evans and Burr (5) in the concentration of vitamin E from wheat germ oil, except that ace-

¹ Freshly distilled, less than 24 hours previously.

DIAGRAM 1
Fresh lettuce leaves, 140 kilos
 |
Dried *in vacuo*, 10 kilos
 |
Extracted with alcohol



tone has been introduced as the first solvent for fractional crystallization.

Acetone-Insoluble Fraction—The granular precipitate which separated from the hot acetone on cooling was dried, and distilled *in vacuo*. Approximately 80 per cent distilled from 200–280°. The fractions are white, waxy materials with melting points from 60–80°. The greater portion is composed of alcohols, but at least one hydrocarbon is also present. Separation of the different constituents is difficult, but some degree of purity can be attained by numerous fractional crystallizations from acetone. The compounds are being studied, and a more complete characterization will be given in a subsequent paper.

Petroleum Ether-Insoluble Fraction—The oily, resinous material was dissolved in ether, and an insoluble white powder was filtered out. The compound is comparatively insoluble in organic solvents. It was recrystallized from large amounts of methyl alcohol, and melted at 240–247° with decomposition. No other definite compound could be obtained from the residues.

Methyl Alcohol-Insoluble Fraction—The precipitate was dissolved in ether and allowed to stand at –5° for several weeks. The crystals of carotene which separated were filtered and dried. A study of some of the properties of carotene has already been reported (9). The filtrate was evaporated to dryness *in vacuo* and dissolved in methyl alcohol. The crystals which separated on standing comprised the major portion of the lettuce sterols. Numerous crystallizations from alcohol yielded a pure phytosterol which melted at 146°; acetate m.p. 126°. More complete studies of the sterols, of which there are certainly two, and possibly more, are in progress.

Petroleum Ether-Soluble Fraction—The vitamin E content of the unsaponifiable lipids was concentrated in this fraction. Vitamin E assays had been made of each fraction in the course of the separation, by determining the minimum amount sufficient for the birth of a litter when fed in a single dose on the day of positive mating. The female rats used were on a vitamin E-free ration, and had previously undergone a typical resorption gestation. The brown oily material solidified at 0° without crystal formation; iodine number, 130 ± 10 . The vitamin E can be markedly concentrated by distillation *in vacuo*. Between 140–150°, at 0.1 mm.

pressure, 60 per cent of the fraction distils as a light yellow, clear oil which solidifies at about 0°.

The vitamin is obtained in the higher boiling fractions, and has shown all the properties described by Evans and Burr (5) for vitamin E from wheat germ oil. The concentrates are being investigated.

92 Per Cent Methyl Alcohol-Soluble Fraction—The antioxygenic activity of the original extract was concentrated in this fraction.

TABLE I
Separation of Antioxidant and Vitamin E between Solvents

Preparation No.	Antioxygenic index*		Minimum dose for normal gestation	
	Petroleum ether-soluble fraction	92 per cent methyl alcohol-soluble fraction	Petroleum ether-soluble fraction	92 per cent methyl alcohol-soluble fraction
			mg.	mg.
12	3.0	17.8	150	>250†
13	1.5	20.0	150	>250†

* The ratio of induction period of a standardized mixture of fats with added antioxidant, to that of the fat mixture alone. 5 mg. of the fraction to be tested were added to 5 gm. of the fat mixture.

† No litters were obtained with 250 mg. doses.

The content of antioxidant of each of the fractions had been assayed by determining the ability of the fraction to prolong the induction period of a standardized mixture of autoxidizable fats. Table I illustrates the separation of antioxidant and vitamin E between the solvents, 92 per cent methyl alcohol and petroleum ether.

No crystals could be obtained by evaporation of solutions in a number of organic solvents, but the antioxidant could be concentrated by distillation *in vacuo*. The details of its isolation are recorded in the following paper.

DISCUSSION

The almost invariable association of vitamin E with antioxygenic material in natural foodstuffs (3) has thus far prevented an understanding of the function of vitamin E, whether it is itself an antioxidant or whether its presence in foods is dependent upon the simultaneous presence of antioxygenic substances which protect

it against oxidation. The successful segregation of vitamin E and antioxidant material so that neither possesses the capacity inherent in the other would seem finally to answer this question.

SUMMARY

The unsaponifiable lipids of lettuce have been fractionated chemically, and to a certain extent physiologically, by the use of organic solvents. Alcohols of high molecular weight, several sterols, and carotene have been isolated; and concentrates of vitamin E and of an antioxidant have been prepared. Several other substances remain to be identified. The vitamin E of lettuce has the same solubilities as that of wheat germ oil. The antioxidant and vitamin E are two distinct entities.

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THE UNSAPONIFIABLE LIPIDS OF LETTUCE

III. ANTIOXIDANT*

By H. S. OLCOTT AND H. A. MATTILL

(From the Biochemical Laboratory, State University of Iowa, Iowa City)

(Received for publication, June 15, 1931)

For almost a century it has been known that certain plant tissues or plant products contain substances that act in a manner to prevent rancidity in fats (4), or to delay the drying of linseed oil (2). The increasing importance of antioxygenic materials in the chemistry and physiology of nutrition has been discussed in earlier papers (3, 6) in which it was pointed out that the permanence of vitamins A and E, especially the latter, seemed to be dependent upon the presence of antioxidants. A study of the unsaponifiable lipids of lettuce was undertaken primarily for the purpose of isolating the antioxygenic material, determining whether it was a substance separate and distinct from vitamin E, and establishing some of its physical and chemical properties.

Preparation

In the preceding paper (9), the methods of preparation and fractionation of the unsaponifiable lipids of lettuce were reported. The antioxygenic activity was found concentrated in the fraction preferentially soluble in 92 per cent methyl alcohol, while the bulk of extraneous material, including vitamin E, remained in the petroleum ether layer. The residue left after removal of the methyl alcohol and water was subjected to distillation *in vacuo* (0.1 to 0.2 mm. pressure). Each fraction was assayed for its antioxidant content and that which had distilled between 165–185° contained the greatest amount. White crystals appeared in this

* The data of this and the preceding paper were reported in part before the Montreal meeting of the American Society of Biological Chemists (Olcovich, H. S., and Mattill, H. A., *J. Biol. Chem.*, **92**, p. xxxi (1931)).

oily fraction on standing. They were purified by crystallization from minimum amounts of chloroform, and proved to be the compound responsible for the antioxygenic activity. About 100 mg. have been obtained, representing 10 kilos of dried leaves. The separation was in no way quantitative.

Physical Properties

The sparkling, white crystals melt at 143° (corrected). If the heating is continued, the melt turns brown at $225\text{--}230^{\circ}$, and is completely charred at 300° . The antioxidant is only slightly soluble in water and petroleum ether, moderately soluble in chloroform and benzene, and soluble in ether, acetone, and dilute alkalis. The solution in alkalis is yellow; concentrated solutions are reddish yellow. Alcohol solutions are optically inactive.

From acetone, the crystals are prisms terminated by pyramids, and are monoclinic. The optical properties are somewhat unusual, and may be used for identification. The negative biaxial crystals have a very high birefringence and index of refraction. The extinction angle, C to cleavage, is 30° , and $2V$ is approximately 70° . There is extreme horizontal dispersion.¹

Chemical Composition

Nitrogen is not present. Carbon and hydrogen were determined by micro combustions,² and the molecular weight by the camphor method of Rast. The probable formula is $C_{13}H_{14}O_5$.

Analysis

3.794 mg. substance: 1.86 mg. H_2O and 8.68 mg. CO_2 .

4.440 " " : 2.30 " " " 10.17 " "

3.213 " " : 1.65 " " " 7.43 " "

$C_{13}H_{14}O_5$. Calculated. C 62.33, H 5.64

Found. " 62.40, " 5.49

" 62.47, " 5.79

" 63.06, " 5.75

2.4 mg. substance: 21.75 mg. camphor, Δ 17.5° .

2.6 " " : 22.3 " " Δ 18.5° .

$C_{13}H_{14}O_5$. Calculated molecular weight, 250; found, 252, 252

¹ We are indebted to Mr. D. C. McCann for the optical analyses.

² We are indebted to Dr. H. T. Clarke for the micro combustions of the antioxidant and its acetyl derivative.

The Liebermann-Burchard test is negative. Bromine is not added. A transient, but definite, blue-green coloration is obtained by adding 1 drop of a dilute solution of ferric chloride to a saturated aqueous solution. Yellow, alkaline solutions may be decolorized by acid, and the antioxidant appears as a flocculent precipitate. Solutions in concentrated sulfuric acid have a light green fluorescence.

The antioxidant is very easily oxidized. Alkaline potassium permanganate, concentrated nitric acid, and ammoniacal silver nitrate are reduced instantly and in the cold.

An acetyl derivative was prepared by heating 7 mg. of the antioxidant with 1 cc. of acetic anhydride (purified by distillation) for 4 hours on a steam bath. The excess acetic anhydride was evaporated in a stream of CO_2 and the residue dissolved in a few drops of petroleum ether. The white crystals which developed on standing were filtered. Yield 6 mg., m.p. 72° .

Analysis

4.510 mg. substance: 2.30 mg. H_2O , 10.05 mg. CO_2 , 0.04 mg. residue.

$\text{C}_{15}\text{H}_{16}\text{O}_6$ (1 acetyl). Calculated. C 61.62, H 5.52

Found. " 61.32, " 5.72

2.5 mg. substance: 23.05 mg. camphor, $\Delta 15^\circ$.

$\text{C}_{15}\text{H}_{16}\text{O}_6$ (1 acetyl). Calculated molecular weight, 292; found, 287

The few mg. used for the micro combustion were slightly impure as indicated by their yellowish cast, and by the trace of residue remaining after burning.

The acetate is insoluble in cold alkalies, but is hydrolyzed, with the production of the typical yellow color, by heating. It has no antioxygenic activity.

Antioxygenic Capacity

The method of assay for antioxygenic capacity has been described before (6). In the preliminary separation outlined in Diagram 1 of the preceding paper (9) 5 mg. samples were added to the standardized mixture of lard and cod liver oil; as the fractions became more concentrated in antioxidant, 1 mg. was sufficient for definite measurement. The prolongation of the induction period measured the efficacy of the added material. Results were interpreted in terms of an antioxygenic index, the ratio of the induction period with added material to that of the fat mixture

without addition. It should be emphasized that this index is not a constant for any given antioxygenic substance; it is dependent also on the character of the fat mixture used, and the temperature. Only when all other conditions are kept constant may the capacity of one antioxidant be compared with that of another in terms of an antioxygenic index. In a fat mixture composed of 5 gm. of lard and 10 drops of cod liver oil (the latter irradiated for 30 minutes to shorten the induction period to a convenient time, 6 to 8 hours), 1 mg. of the lettuce antioxidant had an index of 29 at 70°. That for

TABLE I

Comparison of the Antioxygenic Capacity of Lettuce Antioxidant with That of Synthetic Phenols and Naphthols

1 mg. of antioxidant in 5 gm. of autoxidizable fat (0.02 per cent).

Compounds	Antioxygenic index
Hydroquinone.....	120
Pyrogallol.....	>64
Pyrocatechol....	>55
α -Naphthol.....	31
Lettuce antioxidant.....	29
β -Naphthoquinone....	25
1,8-Dihydroxynaphthalene....	21*

* Determined since the publication of Mattill's work (6).

α -naphthol under similar conditions was 31. Table I compares the lettuce antioxidant with those phenols and naphthols whose indices have been determined under these conditions (6).

DISCUSSION

The antioxygenic action of phenols and the phenolic reactions of the naturally occurring inhibitors of oxidation suggest that these latter are phenol derivatives, but few attempts have been made to isolate them. Indeed the only instance seems to be the work of Bruson, Sebrell, and Vogt (1) who were successful in isolating the natural oxidation inhibitors of crude Hevea rubber from the non-saponifiable constituents. Their most active product appeared to be a derivative of phytosterol and contained one hydroxyl group. Acetylation destroyed its antioxygenic property.

The observations of Moureu and his coworkers (8) upon phenols and phenol ethers, and the more recent studies of phenols (6) of the antioxidant in corn oil (7), and now in lettuce, alike indicate that the inhibiting action of this type of antioxidant resides in the hydroxyl group.

Substances of phenolic nature are not rare in plants. Tannins are widely distributed but they are not fat-soluble and they do not possess antioxygenic activity in fat systems. Thymol and phloroglucinol are found in vegetable oils but they are feeble antioxidants. The mere presence of a hydroxyl group, or even of two of them, does not make an antioxidant. Phenol itself is inactive under the conditions of measurement while α -naphthol has powerful antioxygenic action. Just what structural characteristics an antioxidant must possess cannot yet be stated and it is probable that the various plant tissues do not all synthesize the same one. The extensive study of rubber just referred to revealed at least three different antioxidants, two of which, when analyzed, showed a much smaller oxygen content than the product from lettuce.

The rôle of antioxidants in plants has recently achieved a greater interest than merely that of improving the keeping qualities of vegetable oils. Carotene has long been known to be easily oxidized, and according to Kohl (5) it may be an agent enabling plant tissue to further its process of oxidation. Carotene is a prooxidant (10). With the demonstration that carotene is provitamin A, its permanence in plant tissue by virtue of its association with an antioxidant is increasingly important.

The function of antioxidants in foodstuffs as preservers of vitamin E has been discussed in another connection (3). The identification of the antioxidant as an entity separate from vitamin E (9) may add another item to the list of essential lipids, especially if it can be demonstrated that the activity of the antioxidant is not restricted to the protection of the vitamin in food but extends to the regulation of its physiological activity. The recent experiments of Waddell and Steenbock (11) indicate that the prooxidants (antivitamin) produced by treatment of rations with ferric chloride in ether had a profound effect not only in the ration but also in the body of the rat. The restoration of fertility in female rats which they secured by administering vitamin E in the form of wheat germ oil was due in part to additional vitamin supplies but doubt-

less also to the accompanying antioxidant in wheat germ oil. The demonstration that vitamin E and antioxidant are separate entities will facilitate a clearer exposition of the rôle of each.

SUMMARY

A characteristically crystalline antioxidant has been isolated from the unsaponifiable lipids of lettuce by methods of fractional distillation and crystallization. As indicated by combustion and molecular weight determinations, its formula seems to be $C_{13}H_{14}O_5$. One hydroxyl group, phenolic in character, is present, and an acetyl derivative has been prepared. Physical and chemical properties are outlined, and its possible functions in plant and animal life are briefly discussed.

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ON THE OXIDATION OF LINSEED OIL EMULSIONS IN THE PRESENCE OF HEMATIN AND POTASSIUM CYANIDE

BY G. PAYLING WRIGHT AND MARGARET VAN ALSTYNE

(From the Cancer Commission of Harvard University, and the Department of Physical Chemistry in the Laboratories of Physiology, Harvard Medical School, Boston)

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Certain oxidations of biological interest proceed much more actively in the presence of iron derivatives, among them the hemin compounds. The acceleration of the oxidation of linseed oil emulsions on addition of hemin derivatives has been described by Robinson (1924) and of oleic acid and certain other fatty acids by Kuhn and Meyer (1929). The slight inhibitory action of cyanide on these reactions was also shown by these investigators.

On the other hand the few observations on the oxidation of amino acids have yielded apparently contrary results. Krebs (1929) found that the oxidation of cysteine in the presence of hemin compounds was inhibited by low concentrations of cyanide. These observations will be discussed in more detail later. Harrison (1924) also studying cysteine found that hemin, while less active as a catalyst than inorganic iron, was less sensitive to the presence of cyanide.

Recent studies (Keilin, 1929; Warburg, 1928) have directed attention to the importance of hemin compounds in cellular oxidations. Since the remarkable inhibitory influence of cyanide on tissue respiration has been associated with its reactivity with iron compounds, further examination of the action of cyanide on catalysis by hemin compounds is clearly wanted. This paper records observations on the oxidation of linseed oil in the presence of certain hemin compounds, more especially cyanide-hematin itself.

Methods—The linseed oil emulsion used in all experiments con-

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tained 1 cc. of oil in 250 cc. of 1 per cent Na_2CO_3 . The mixture was emulsified by vigorous shaking for several minutes. The emulsions were almost always made just before use, since after remaining for several hours at room temperature they oxidized spontaneously at a measurable rate. The oil used had a specific gravity of 0.928 at 24° , an iodine number of 171, and contained less than 0.1 mg. of nitrogen per gm.

Two specimens of hemin were used: Preparation A from the Eastman Company, and Preparation B made in this laboratory by Dr. T. L. McMeekin to whom we are greatly indebted. The

TABLE I
Oxidation of Linseed Oil Emulsions in Presence of Pyridine-Hematin

Tube No.	1	2	3
	cc.	cc.	cc.
Linseed oil emulsion	2	2	2
Water	1		
Pyridine-hematin*		1	1
Potassium cyanide, 0.1 M			0.2

Experiment No.	Hemin	C.mm. O_2 consumed per cc. mixture in 2 hrs.		
1	Preparation A	1	156	152
2	" "	Nil	144	152
3	" "	"	161	134
4	" B	"	100	96
5	" "	7.1†	105	101

* The pyridine-hematin solution contained 0.25 M pyridine.

† This linseed oil emulsion was made several hours previous to its use.

solutions of hemin derivatives used contained, unless otherwise stated, 40 mg. of hemin per 100 cc. Solution of the crystals of hemin in N NaOH, and reprecipitation with an equal volume of N HCl, made no perceptible difference to its subsequent catalytic properties. The nomenclature of the hemin compounds here used is taken from the table in the paper by Krebs (1929).

Oxidation rates were measured in a Barcroft-Warburg apparatus. The temperature employed was 37° . The volumes of solutions used varied in different experiments and can be found by examining the various tables.

Oxidation of Linseed Oil Emulsions in Presence of Pyridine-Hematin

The experimental results for the oxidation of oil emulsions in the presence of alkaline solutions of hemin and pyridine, with and without the addition of potassium cyanide, will be found in Table I. It is evident from them that cyanide (in roughly 30 times the molar concentration of the hemin present) exercises little or no check on the oxidation reaction, a result in agreement with that of Robinson, who stated that the inhibitory effect of cyanide "was found to be entirely absent in the case of hemin."

TABLE II

Oxidation of Linseed Oil Emulsions in Presence of Cyanide-Hematin

Tube No.	1	2	3	4
	cc.	cc.	cc.	cc.
Linseed oil emulsion.....	2	2	2	2
Water.....	1			
Pyridine-hematin*.....		1		
Cyanide-hematin (0.06 M KCN) ..			1	
" (0.15 " ") ..				1

Experiment No.	Hemin	C.mm. O ₂ consumed per cc. mixture in 2 hrs.			
1	Preparation B	7.1†	105	112	
2	" A	Nil	161	137	
3	" B	"	159	162	
4	" "	1	151	143	
5	" "	1		145	161

* The pyridine-hematin solution contained 0.25 M pyridine.

† This linseed oil emulsion was made several hours previous to its use.

Oxidation of Linseed Oil Emulsions in Presence of Cyanide-Hematin

The solution of 10 mg. of hemin in 25 cc. of KCN (0.06 M or stronger) results in the formation of cyanide-hematin, the proportion of the three substances present being calculable from the dissociation constant for the compound. We are greatly indebted to Mr. A. M. Pappenheimer for informing us in a personal communication that for hematin in the presence of a borate-tartrate buffer of pH 9.1, he has found the constant to be about 5×10^{-4} . This would imply that in 0.02 M KCN, slightly more than 1 per cent, and in 0.05 M KCN, less than 0.25 of 1 per cent of hematin

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would be in the free condition. The results of several experiments in which hematin was dissolved in potassium cyanide solutions will be found in Table II.

These experiments show that the hemin in the presence of relatively strong concentrations of potassium cyanide and in the absence of pyridine remains effective for the oxidation of the oil emulsion. In this connection it is interesting that Conant and Tongberg (1930) found that the addition of potassium cyanide to hematin, dissolved in a borate-tartrate buffer of pH 9.1, increased its oxidation potential.

TABLE III

Results of Variations in Concentration of KCN on Oxidation Rate of Oil Emulsions

Flask No	Hemin Preparation B		Potassium cyanide		Color of solution	C.mm. O ₂ consumed per cc. linseed oil emulsion (in 2 hrs.)	
						Experiment 1	Experiment 2*
	mg.	mols	mg.	mols			
1	10	0.00015	2.5	0.00038	Brown	59	60
2	10	0.00015	5.0	0.00076	"	68	
3	10	0.00015	10.0	0.00152	"	69	67
4	10	0.00015	20.0	0.00303	" to red		66
5	10	0.00015	30.0	0.00455	" " "	68	
6	10	0.00015	50.0	0.00757	Brownish red		66
7	10	0.00015	100.0	0.01515	Red	78	73

* 1 cc. from each of Flasks 1 to 7 was added to 2 cc. of the oil emulsion and the resulting oxygen consumption is shown in this column.

Addition of dilute alkaline cyanide solutions to hemin appears to give rise to two compounds, one of which may be hematin alone in alkaline solution and the other cyanide-hematin, or both may be analogous with the two cyanide hemochromogens described by Anson and Mirsky (1928). At low concentrations of cyanide a soluble brown pigment was formed, at high concentrations a cherry-red one, and at intermediate ones both pigments were present together in varying proportions. The relative concentrations of hemin and cyanide producing these compounds can be seen from Table III. In Table III will also be found the results of variations in the concentration of potassium cyanide on the oxidation rate of the oil emulsions.

This experiment shows that increasing the concentration of cyanide, within this range (for higher ranges see Table II), does not decrease the oxidation rate of the emulsion. On the contrary, it results in a slight but progressive increase, though necessarily more of the hematin is present in the form of cyanide-hematin at the higher cyanide concentrations.

Oxidation of Linseed Oil Emulsions in Presence of Animal Charcoal

The oxidation of amino acids and certain other organic compounds in the presence of charcoal has been studied by Warburg

TABLE IV
Oxidation of Linseed Oil Emulsions in Presence of Animal Charcoal

Tube No.....	1	2	
	cc.	cc.	
Linseed oil emulsion.....	2	2	
Animal charcoal suspension.....	1	1	
Potassium cyanide, 0.025 M.....		0.2	
Experiment No.	C.mm. O ₂ consumed per cc. mixture in 2 hrs.		Percentage inhibition
1	27.6	11.5	58
2	38.8	11.2	71
3	30.8	7.9*	75
4	13.4†	4.4	67

* 0.2 cc. of potassium cyanide, 0.05 M, was used in this experiment.

† A different specimen of linseed oil was used in this experiment.

and his collaborators (1928) and by Rideal and Wright (1925, 1926). Warburg has shown that oxidations in iron-containing charcoal suspensions are very sensitive to cyanide. Consequently were the oxidation of the oil emulsions in the presence of animal charcoal inhibited by cyanide, it would make improbable the possibility that the emulsion itself contained some constituent which, by combining with the cyanide, reduced its effective concentration. The results of several experiments will be found in Table IV. The animal charcoal was used in a 20 per cent water suspension, each Barcroft-Warburg flask receiving 200 mg. This ratio of charcoal to substrate is about that used by Warburg and Negelein (1920) in the oxidation of cystine and other amino acids.

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Control experiments showed that the animal charcoal and the oil emulsion separately had negligible oxidation rates both in the presence and absence of cyanide. When present together, however, the charcoal greatly accelerates the oxidation of the oil emulsion. This acceleration is considerably inhibited by the presence of potassium cyanide. These observations make it very improbable that the failure of the cyanide to inhibit the catalytic action of the hematin in the previous experiments was due to its combination with and consequent neutralization by the substrate. The cyanide is free to associate either with the charcoal, as in these experiments, or with the hematin as in the previous ones.

TABLE V

Influence of Varying Concentrations of Pyridine-Hematin on Oxidation Rate of Linseed Oil Emulsions

Tube No	1	2	3	4	5	6
Linseed oil emulsion, cc.	2 00	2 00	2.00	2.00	2 00	2 00
Pyridine-hematin solution, cc....	1 00	0.50	0 25	0.13	0.06	Nil
Water, cc.....	Nil	0.50	0.75	0.87	0 94	1.00
Hemin per 100 cc. in final pyridine-hemin solution added, mg.....	40 0	20 0	10 0	5.0	2 5	Nil
O ₂ consumed per cc. mixture in 2 hrs., c.mm.....	131	92	70	45	31	1

Influence of Varying Concentrations of Pyridine-Hematin on the Oxidation Rate of Linseed Oil Emulsions

The concentration of the pyridine-hematin in the previous experiments being possibly in excess of that necessary to maintain the oxidation rate at the figure recorded, it was progressively diluted to determine at what concentration the rate became adversely affected. For, should the oxidation rates hitherto recorded require merely a trace of the hematin compound, such a trace still present in the cyanide-hematin might in reality remain the effective catalyst. Being masked by the preponderating concentration of the cyanide-hematin it might readily pass unrecognized. In those experiments recorded in Table I, for example,

the ratio of cyanide-hematin to pyridine-hematin plus free hematin might be 99 to 1, yet the small quantity of the latter might be responsible for the undiminished oxidation rate. In the experiments with cyanide-hematin alone (see Tables II and III) this possibility is almost excluded, for while the ratio of cyanide-hematin to free hematin varies widely the oxidation rate remains substantially the same.

This possibility is also rendered less probable should a reduction of the concentration of the pyridine-hematin affect the rate of the

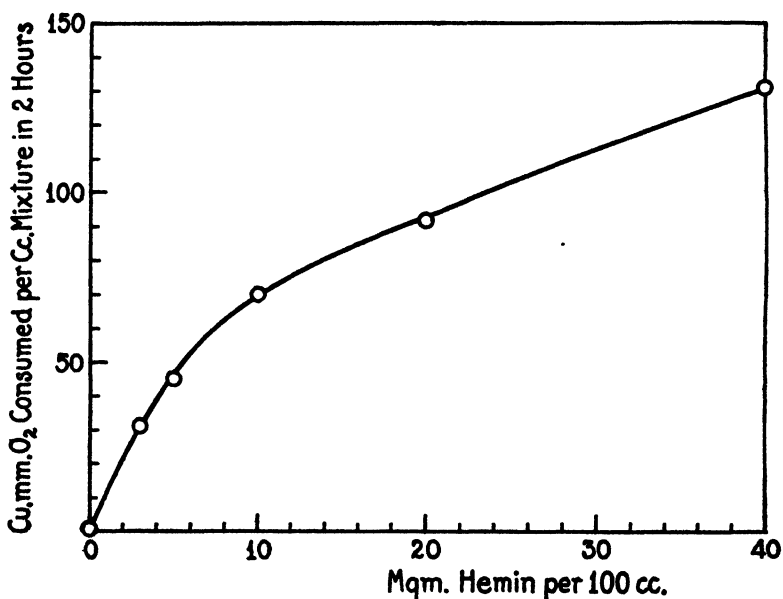


CHART I

reaction. The results of an experiment in which the pyridine-hematin was progressively diluted with water will be found in Table V and Chart I.

This experiment definitely shows that a reduction of the concentration of the hematin below that previously used lowers the oxidation rate. From this it seems clear that had part of the hematin used in the experiments given in Table I been inactivated by the addition of cyanide the oxidation rate of the emulsion would have dropped. Hence it seems correct to infer that, since the

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oxidation rate did not drop and cyanide-hematin was certainly formed, the compound of potassium cyanide with hematin acted as effectively in promoting oxidation as hematin either free or combined with pyridine.

While these experiments render highly improbable they do not entirely exclude the possibility of an impurity being present which combined the properties of forming a highly active compound with hematin with a great resistance to its displacement by potassium cyanide. Fortunately owing to the kindness of Professor J. B. Conant we had an opportunity to study the effect of two specimens

TABLE VI
Comparison of Mesohematin and Hematin on Oxidation Rate of Linseed Oil Emulsions

Tube No.	1	2	3	4	5
	cc.	cc.	cc.	cc.	cc.
Linseed oil emulsion.	2	2	2	2	2
Water.....	1				
Pyridine-hematin.....		1			
Pyridine-mesohematin.....			1		
KCN-mesohematin.....				1	
KCN (0.06 M).....					1
Experiment No.	Mesohemin		C mm. O ₂ per cc. mixture in 2 hrs.		
1	Preparation A		2.7	138	85
2	" B		2.3	129	155
3	" A		Nil	122	73
				146	143
				132	Nil

of mesohemin on the oxidation of the linseed oil emulsions. These substances, prepared by the reintroduction of iron into mesoporphyrin, would be unlikely to contain such an impurity after the succession of processes to which they had been subjected. Both preparations were made up with pyridine and potassium cyanide in the same concentrations as were the original hematin solutions. The linseed oil employed was the same as had been previously used. The results will be found in Table VI.

Except mesohematin, Preparation A, in pyridine water, both specimens appeared to accelerate the oxidation as much as the original hematin. Such a close agreement with four different

hematin preparations (McMeekin's and Eastman's hematin and Conant's two mesohematin) is strongly opposed to the oxidation being dependent on the presence of a highly active impurity.

Oxidation of Cystine in Presence of Hematin

In other studies it was found that the oxidation of cystine in alkaline solution is accelerated by cyanide-hematin but not by

TABLE VII
Oxidation of Cystine in Presence of Hematin

Tube No.....	1	2	3	4	5
	cc.	cc.	cc.	cc.	cc.
Cystine (0.0125 M).....	2	2	2	2	2
Water.....	1				
Pyridine-hematin.....		1	1		
Cyanide-hematin.....				1	
KCN (0.062 M).....					1
" (0.10 M).....			0.2		
Experiment No.	C.mm. O ₂ consumed per cc. mixture in 9 hrs.				
1	1	4	49	71	12
2	4	3	47	64	9
3	4	2	49	63	9
Mean.....	3	3	48	66	10

The cystine was dissolved in 1 per cent Na₂CO₃ solution. The pyridine-hematin contained 40 mg. of hemin and 2 cc. of pyridine in 100 cc. The cyanide-hematin contained 40 mg. of hemin in 100 cc. of 0.062 M KCN solution.

hematin dissolved in pyridine water. The results of these experiments are to be found in Table VII.

DISCUSSION

Keilin and Warburg have indicated that hemin derivatives probably play an important part in cellular oxidations. For this reason the catalytic oxidation of organic compounds by hemin derivatives, together with the degree to which these reactions can, like respiration, be inhibited by cyanide, has attained greater importance. Until now too few observations on these reactions have been recorded

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to allow of any satisfactory generalizations, though Kuhn and Meyer (1929) have suggested that the ability of cyanide to inhibit these oxidations is largely dependent on the chemical nature of the substrate. On the basis of observations of the failure of cyanide to inhibit the oxidation of linseed oil and oleic acid in the presence of pyridine-hematin, they suggested that cyanide might inhibit the oxidation of carbohydrates yet have little or no influence on that of fats. In this way they explained the results of Dixon and Elliot (1929), who had observed in certain tissues poisoned by

TABLE VIII
Cyanide Inhibition for Oxidation of Amino Acid Cysteine in Presence of Hemin and Its Derivatives
Compiled from Tables V and XVIII in Krebs' paper.

Flask No*... Table No. . .	1 V	2 V	3 XVIII	4 XVIII
Borate buffer; pH.	0.25 M; 10.3	0.25 M; 10.3	0.25 M; 10.3	0.25 M; 10.3
Pyridine, M. . . .	3.0	3.0	3.0	3.0
Hemin, M.	0.77×10^{-5}	0.77×10^{-5}	0.60×10^{-5}	0.60×10^{-5}
Cysteine M.	3.8×10^{-2}	3.8×10^{-2}	5.0×10^{-2}	5.0×10^{-2}
FeSO ₄ , M.	1.8×10^{-6}	Nil	1.8×10^{-6}	1.8×10^{-6}
Cyanide, M.	Nil	"	Nil	10.2×10^{-5}
Temperature, °C.	10	10	20	20
Rate of oxidation, c.mm. O ₂ per hr..	111.3†	27†	109	34.4

* Each flask contained 2 cc. of the above solutions upon which the oxidation rate was determined.

† In these flasks the oxygen consumption was measured for only 20 minutes, but the rate is here expressed as per hour.

cyanide that a reduced respiration persisted (on average about 40 per cent of the normal) together with a simultaneous lowering of the respiratory quotient.

While it seems clear that the oxidation of certain fatty acids in the presence of hemin is unaffected by cyanide, Krebs (1929) has described a cyanide inhibition for the oxidation of the amino acid cysteine in the presence of hemin and some of its derivatives. Since his observations are of considerable interest in relation to Kuhn and Meyer's suggestion, and on account of the rather com-

plicated nature of his system, a summary table of some of his results is reproduced in Table VIII.

The closely similar concentrations of reagents used in these experiments make their semiquantitative comparison justifiable. It should be especially noted that only Flask 2 contained hemin without the accompaniment of inorganic iron. Its contents absorbed oxygen at only 24 per cent of the rate of that in its companion Flask 1, which only differed by the addition of FeSO_4 (1.8×10^{-6} mols per liter). (Warburg and Negelein (1928) first showed that inorganic iron can act synergistically with hemin in catalyzing such oxidation.) On comparison of Flasks 3 and 4 it can be seen that in spite of cyanide in Flask 4, its contents still absorbed oxygen at a rate not dissimilar to that of Flask 2, and at about 30 per cent of the rate of that of its companion, Flask 3, which contained no cyanide.

It would seem in better accord with these observations to regard the cyanide inhibition as affecting mainly, if not exclusively, the synergistic action of the ferrous sulfate, a view which is further supported by reference to Table XVII of Krebs' paper. Unfortunately we could find no experiment described in which the action of cyanide upon the oxidation by hemin or a hemin derivative was examined in the absence of inorganic iron. Without this direct experiment it is hardly possible to accept the conclusion that cyanide inhibits the hemin in this reaction.

To the present, therefore, the few recorded observations appear to show that neither the oxidations of linseed oil studied by Robinson and by ourselves, nor of certain fatty acids studied by Kuhn and Meyer, nor cystine studied by ourselves, nor, as it seems to us, cysteine studied by Krebs, appear to be affected much, if at all, by the presence of cyanides, yet in all the instances hemin or a hemin derivative was used as the catalyst. It should be recalled, however, that these experiments were carried out under conditions very different from those occurring in the living cell, and the results can only be used with the utmost caution in interpreting the action of cyanide in inhibiting cellular respiration.

SUMMARY

1. The observations of Robinson (1924) on the acceleration of the oxidation of linseed oil emulsions by hemin, together with the

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failure of potassium cyanide to inhibit the reaction, have been repeated and confirmed.

2. Cyanide-hematin accelerates the oxidation of linseed oil emulsions almost as effectively as does pyridine-hematin. Wide variations in the concentration of cyanide have little influence on the rate of the reaction.

3. Linseed oil emulsions have their oxidation accelerated by the addition of animal charcoal. This oxidation is markedly inhibited by the presence of potassium cyanide.

4. Hematin dissolved in pyridine water (0.25 M) has no influence on the oxidation rate of cystine. Addition of a small quantity of potassium cyanide to the solution induces a rapid oxidation. Cyanide-hematin in the absence of pyridine similarly accelerates the oxidation of cystine.

We should like to express our indebtedness to Professor J. B. Conant for his advice and criticism.

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THE ORIGIN OF GLUCOSE LIBERATED BY EPINEPHRINE IN DEPANCREATIZED ANIMALS

By JESSE L. BOLLMAN, FRANK C. MANN, AND
CHARLES M. WILHELMJ

(From the Division of Experimental Surgery and Pathology, The Mayo Foundation, Rochester, Minnesota)

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The effect of epinephrine on the mobilization of glucose has been reviewed by Rapport (8). The main points in his summary of the subject may be outlined as follows: Epinephrine produces hyperglycemia and glycosuria in animals except when the glycogen is extremely low. Larger amounts of glucose are excreted when the glycogen content is high, and with continued injections of epinephrine there is a decrease in the amount of sugar excreted. Almost similar observations have been made on the phlorhizinized animal and on the depancreatized animal. Chaikoff and Weber (3) used depancreatized dogs to study the source of the sugar found in the urine following injections of epinephrine. They found that well nourished depancreatized dogs excrete more glucose following injections of epinephrine than can be accounted for by the formation of glucose from the protein and glycerol portion of the fat metabolized during that period. The glycogen content of the liver of similar diabetic animals was not sufficient to account for more than a fraction of the glucose excreted. In considering the possibility of muscle glycogen as a source of glucose under these conditions, Chaikoff and Weber called attention to the work of Mann and Magath (7), Bollman, Mann and Magath (2), Soskin (9), and others which they interpreted as establishing that muscle glycogen cannot contribute directly to the sugar of the blood. After removal of the liver there is no change in the content of muscle glycogen, sugar, or lactic acid in the blood produced by the injection of epinephrine. For this reason it was concluded that the glucose obtained did not come from the glycogen of the muscles

and by exclusion must have been formed from fatty acids. Unfortunately, the glycogen content of the animals used was not determined, but it was calculated that even if the muscle glycogen was considered as a source of glucose, two of the animals used still had an excess of sugar to be accounted for, but the others of the series showed a loss by this method. Accordingly, Rapport (8) and others have suggested that the glucose formed may have been derived from the muscle glycogen, possibly by conversion of lactic acid, as is indicated by the work of Cori and Cori (4). In view of the significance of this work we have conducted similar experiments, with the exception that the glycogen and glucose content of the blood, liver, and muscles was determined before and after injection of epinephrine.

Methods

Depancreatized animals were maintained for about a month after operation until they appeared to be in excellent condition. During this time they were fed twice daily with a mixture of meat, cracker crumbs, syrup, and lard to which 100 gm. of fresh pancreas were added. After each meal from 20 to 60 units of insulin, depending on the amount of food consumed, were given subcutaneously. The last meal and insulin were given in the evening, and the next morning the animals were catheterized and placed in metabolism cages with free access to water. 24 hour specimens of urine were collected for the first 2 days, and two 12 hour specimens were collected the 3rd day.

On the morning of the 4th day after the withdrawal of insulin and of fasting, the animals were catheterized and a specimen of blood was obtained from the jugular vein. Under ether anesthesia specimens of liver and muscle were obtained with aseptic technique. Portions of the extensor and adductor group of muscles were taken from one side and a portion of the pectoral muscle was taken from the opposite side. At the conclusion of this operation from 0.2 to 0.5 mg. of epinephrine in a 50 cc. solution of sodium chloride was injected intravenously, and 0.5 to 1.0 mg. was given subcutaneously. This procedure was repeated at the end of 2 hours, and subcutaneous injections of epinephrine were continued at intervals of 2 hours for the entire 12 hour period. 100 cc. of Fischer's solution were injected intravenously every 2

hours, because this seemed necessary to produce ample diuresis. The animals were catheterized twice at intervals of 6 hours, and the specimens of urine were kept separate. Immediately after the withdrawal of the last specimen of urine a specimen of blood was obtained, anesthesia was induced by an intravenous injection of sodium isoamylethylbarbiturate (sodium amytal), and specimens of liver and muscle were removed. The specimens of muscle removed at this time were taken from the opposite side and corresponded to the specimens first removed. At the conclusion of the experiment the animal was sacrificed by additional anesthetic and was weighed. The organs were then removed and the muscular tissue of the body was dissected and weighed. This was regarded as the weight of the muscles although fascia and some connective tissue were included, but the portions of muscular tags remaining attached to the bones were not included.

The glucose content of the urine was determined by the method of Benedict, total nitrogen by the usual Kjeldahl method, total ketone bodies by the method of Behre and Benedict (1), and lactic acid by the method of Friedemann and Kendall (6). The determination of blood sugar was by the method of Folin (5), and the lactic acid of the blood by the method of Friedemann and Kendall (6).

The glucose content of the liver and muscles was determined as the reducing power (Folin) of a cold alcohol extract of the tissue in the following manner. Immediately after removal of the specimen of tissue, approximately 1 gm. samples were weighed to the nearest mg. and immersed in 20 cc. of cold 70 per cent alcohol. The tissue was then macerated with scissors and extracted twice with this volume of alcohol. The filtrate was concentrated by evaporation and precipitated with sodium tungstate and sulfuric acid. After dilution to volume the reducing power was determined by the method of Folin. An attempt was not made to eliminate reducing substances other than glucose since the figure obtained before the injection of epinephrine is compared to that obtained after injection.

The glycogen content of the liver and muscles was determined by the method of Pflüger. Immediately after the removal of the tissue portions of approximately 1 gm. were rapidly weighed to the nearest mg. and placed in centrifuge tubes containing 1 cc. of

60 per cent potassium hydrate at 100°. The specimens of muscle were dissected almost free from connective tissue and fat before removal, and an effort was not made to free the specimens from extraneous tissue after removal. After hydrolysis of the specimen in potassium hydrate at 100° for 1 hour, glycogen precipitation was

TABLE I

Data for Dog 1, Depancreatized November 10, 1930

Last feeding and insulin injection (40 units) was at 5 p.m., December 12, 1930.

Specimen of urine		Nitrogen	Glucose	Total ketone bodies	D. N	Extra glucose, D: N=2.8	Weight	Epinephrine
<i>hrs</i>	<i>day</i>	<i>gm.</i>	<i>gm.</i>	<i>mg.</i>			<i>kg.</i>	<i>mg</i>
24	1st	7.44	9.03	22	1.23	0	8.0	
24	2nd	6.24	31.75	66	5.10	14.30	7.9	
12	3rd	3.00	21.42	57	7.20	13.03	7.8	
12	4th	1.45	3.08	55	2.13	0	7.5	
6	4th	1.14	10.05	75	8.88	6.87	7.3	4.4
6	4th	1.23	5.47	83	4.35	2.03		4.4

Tissue	Total weight	Carbohydrate balance							
		Before epinephrine				After epinephrine			
		Glucose	Glycogen	Body glucose	Body glycogen	Glucose	Glycogen	Body glucose	Body glycogen
	<i>gm.</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>gm.</i>	<i>gm.</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>gm.</i>	<i>gm</i>
Muscle.....	2900		460		13.30		29		0.84
Liver.....	370		168		0.62		148		0.55
Blood.....	650	400		2.60		644		4.18	
Total.....				2.60	13.92			4.18	1.39

The urine contained 8.90 gm. of "extra" glucose; the muscle, liver, and blood lost 10.96 gm.; unaccounted for, -2.06 gm.

accomplished in 66 per cent alcohol and allowed to stand overnight. The glycogen separation was accomplished by centrifugation and the precipitate washed with alcohol and with ether in the same way. Hydrolysis of the glycogen was carried out by the addition of 10 cc. of dilute hydrochloric acid at 100° for 1 hour.

The glucose content of the solution was then determined after neutralization and dilution to the volume necessary for use, similar to blood filtrate after the method of Folin. The figures for glycogen recorded in Tables I to IV represent the glucose equivalent of glycogen. The figures given for liver glycogen represent the

TABLE II

Data for Dog 2, Depancreatized September 15, 1930

The last feeding and insulin injection (40 units) was at 4 p.m., November 2, 1930.

Specimen of urine		Nitrogen	Glucose	Total ketone bodies	D. N	Extra glucose, D: N = 2.8	Weight	Epinephrine
hrs.	day	gm.	gm.	mg.			kg.	mg.
24	1st	5.45	32.10	23	5.90	16.75	10.2	
24	2nd	4.24	23.84	73	5.63	11.98	9.8	
12	3rd	3.25	8.63	117	2.66		9.5	
12	3rd	2.76	7.92	340	2.88	0.19	9.0	
6	4th	1.24	9.81	186	6.92	5.83	9.1	3.5
6	4th	1.92	12.02	490	6.30	6.64		5.0

Tissue	Total weight	Carbohydrate balance									
		Before epinephrine					After epinephrine				
		Glucose	Glycogen	Body glucose	Body glycogen	Body carbohydrate	Glucose	Glycogen	Body glucose	Body glycogen	Body carbohydrate
		gm.	mg per cent	mg per cent	gm.	gm.	gm.	mg per cent	mg per cent	gm.	gm.
Muscle.....	3820	196	299	7.48	11.40	18.88	194	24	7.40	0.92	8.32
Liver.....	302	266	60	0.80	0.18	0.99	218	61	0.66	0.19	0.85
Blood.....	802	245		1.96		1.96	264	0	2.11		2.11
Total.....				10.24	11.58	21.83			10.17	1.10	11.27

The urine contained 12.47 gm. of "extra" glucose; the muscle, liver, and blood lost 10.56 gm.; unaccounted for, +1.92 gm.

average of four to eight determinations which in this series showed less than 3 per cent deviation from the average obtained. The figures given for muscle glycogen represent the average of at least three different muscles with six to twelve determinations. Duplicate determinations of muscle glycogen checked very closely,

and the different muscles used prior to the injection of epinephrine did not show more than 10 per cent deviation from the average. After epinephrine injection the deviation from the average was much greater (50 per cent), but it may be noted that all of these

TABLE III

Data for Dog 3, Depancreatized December 23, 1930

The last feeding and insulin injection (20 units) was at 4 p.m., January 13, 1931.

Specimen of urine		Nitrogen	Glucose	Total ketone bodies	D N	Extra glucose, D N=2.8	Weight	Epinephrine
<i>hrs.</i>	<i>day</i>	<i>gm.</i>	<i>gm.</i>	<i>mg.</i>			<i>kg.</i>	<i>mg.</i>
24	1st	3.47	12.47	23	3.6	2.65	5.9	
24	2nd	2.90	16.80	44	5.8	8.68	5.4	
12	3rd	2.01	10.40	113	5.17	4.75	5.1	
12	3rd	2.10	6.22	157	2.96	0.34	5.1	
6	4th	1.35	6.92	37	5.12	3.14	5.1	2.0
6	4th	1.11	3.20	28	2.89	0.10		1.5

Tissue	Total weight	Carbohydrate balance									
		Before epinephrine					After epinephrine				
		Glucose	Glycogen	Body glucose	Body glycogen	Body carbohydrate	Glucose	Glycogen	Body glucose	Body glycogen	Body carbohydrate
	<i>gm.</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Muscle.....	2000	162	196	3.25	3.92	7.17	170	32	3.40	0.64	4.04
Liver.....	270	250	58	0.68	0.16	0.83	340	100	0.92	0.27	1.19
Blood.....	450	380		1.71		1.71	330		1.48		1.48
Total.....				5.64	4.08	9.71			5.80	0.91	6.71

The urine contained 3.24 gm. of "extra" glucose; the muscle, liver, and blood lost 3.00 gm.; unaccounted for, + 0.24 gm.

figures represent only minimal quantities of glycogen so that the total error is not of great significance in the complete experiment.

Results

The urine excreted during the period when epinephrine was given showed an excess of sugar that could not be accounted for by the

small increase in nitrogen excreted. The lactic acid of the urine was less than 100 mg. for the period and will be disregarded, except as it might indicate an increased transport of lactic acid. The glycogen content of the liver showed little change and re-

TABLE IV
Data for Dog 4, Depancreatized December 23, 1930

The last feeding and insulin injection (40 units) was at 8 p.m., January 25, 1931.

Specimen of urine		Nitrogen	Glucose	Total ketone bodies	D. N	Extra glucose, D N=2.8	Weight	Epinephrine
hrs.	day	gm.	gm	mg.			kg.	mg.
24	1st	5.46	0	0	0	0	11.1	
24	2nd	4.76	27.68	56	5.81	14.38	9.7	
8	3rd	2.26	22.99	48	10.19	16.68	9.2	
16	3rd	3.61	17.43	517	4.82	7.33	9.0	
6	4th	1.59	9.30	176	5.83	4.84	8.9	3.0
6	4th	1.97	10.76	237	5.48	5.32		2.0

Tissue	Total weight	Carbohydrate balance									
		Before epinephrine					After epinephrine				
		Glucose	Glycogen	Body glucose	Body Glycogen	Body carbo- hydrate	Glucose	Glycogen	Body glucose	Body Glycogen	Body carbo- hydrate
		gm.	mg. per cent	mg per cent	gm.	gm.	gm.	mg. per cent	mg per cent	gm.	gm.
Muscle.....	3960	161	356	6.38	14.08	20.46	184	25	7.29	0.99	8.28
Liver.....	460	322	79	1.48	0.36	1.84	458	91	2.13	0.42	2.55
Blood.....	800	300		2.40		2.40	296		2.37		2.37
Total.....				10.26	14.44	24.70			11.79	1.41	13.20

The urine contained 10.16 gm of "extra" glucose; the muscle, liver, and blood lost 11.506 gm.; unaccounted for, -1.35 gm.

mained at a low level; the glucose content was more variable. Two experiments showed a definite increase not related to an increase in the sugar of the blood, but a third experiment showed a slight decrease. The glucose content of the muscles was similar at the beginning and end of each experiment. The glycogen

content of the muscle at the beginning of the period when epinephrine was given was 460, 299, 196, and 356 mg. for each 100 gm. of tissue. After 12 hours under the influence of epinephrine these values fell to 29, 24, 32, and 25 mg. Consideration of the mass of muscle shows a definite loss of a large amount of glycogen of 12.46, 10.48, 3.28, and 13.09 gm. in the different experiments. The urine contained 8.90, 12.47, 3.24, and 10.16 gm. of glucose in addition to that which could be accounted for by the nitrogen eliminated during that period, the ratio between the dextrose and the nitrogen of 2.8 being used. The figures obtained for the changes in concentration of glucose in the blood, muscle, and tissues indicate that 1.58, 0.08, 0.17, and 1.53 gm. of glucose were retained in the body more than that present at the beginning of the period when epinephrine was given. From these figures it appears that the amount of extra glucose formed in the depancreatized animal under the influence of epinephrine is similar to the amount of glycogen that disappears from the muscles.

DISCUSSION

The results of these experiments reveal that epinephrine causes a marked decrease in the glycogen content of the muscles of depancreatized dogs. Since the amount of extra glucose which appears in the urine corresponds closely to the amount of glycogen lost from the muscles, this glycogen may be considered as the source of glucose under these conditions. Evidence was not obtained of additional excretion of glucose which could have been derived from fatty acids or other material.

We have other data which demonstrate that the action of epinephrine is similar in normal dogs. Several hours after injection the glycogen content of the liver is not materially changed, but the glycogen content of the muscles is greatly decreased. If glucose is given with epinephrine the glycogen content of the liver may increase greatly, but the glycogen content of the muscles continues to decrease. In the absence of the liver, however, there is not a definite decrease of the glycogen content of the muscle due to epinephrine, nor is there an increase in the lactic acid content of the blood or urine as occurs in the normal dog. The glycogen content of the muscles may increase in the hepatectomized animal in the presence of epinephrine if glucose is administered.

SUMMARY

Depancreatized animals were maintained in good condition by large doses of insulin and appropriate feeding. 4 days after the withdrawal of insulin, epinephrine increased the glycosuria beyond that which could be accounted for by the conversion of protein to glucose. At the end of a 12 hour period of administration of epinephrine there was little change in the glycogen or glucose content of the liver. The glucose content of the blood and muscles was not materially altered. There was a marked decrease in the glycogen content of the muscles, which in these experiments was reduced to very low values (20 to 30 mg. for each 100 gm. of muscle). In all experiments the glucose found in excess of the ratio between the dextrose and the nitrogen of 2.8 was almost identical in amount with the loss of glycogen from the muscle. Evidence of formation of glucose from other substances (fat) was not obtained.

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THE CALCIUM CONTENT OF THE BODY IN RELATION TO THAT OF THE FOOD*

BY H. C. SHERMAN AND L. E. BOOHER

(From the Department of Chemistry, Columbia University, New York)

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The purpose of the investigation here reported was to study further the calcium requirement in nutrition and the application and interpretation of the so called law of the minimum when calcium is the limiting factor of the food, by means of quantitative determinations of the relationship of the calcium content of the body, and its rate of increase during growth, to the calcium content of the food.

The necessity for an adequate supply of certain inorganic elements in the food of both plants and animals was fully appreciated by von Liebig as early as 1843. As a result of his observations on the relationship of growth to the mineral composition of the food supply, von Liebig expounded the principle of the so called law of the minimum, which may be briefly stated as follows: That growth essential which is furnished by the food supply in minimum amount (relative to the need for growth at a normal rate) will thereby determine the rate of growth, the organism growing only to the extent that it can grow to the normal composition of its kind.

The application of this general principle as a guide in the study of the calcium requirement in nutrition presents two rather formidable complications: First, the question whether we are to regard the bones as having a normal calcium phosphate content for their kind, or as organs in which a reserve supply of calcium phosphate may be stored if a surplus of these elements is supplied by the food. A second complication is the fact that, as regards calcium, "the normal composition of its kind" changes very markedly with the age of the animal.

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The body of a normal new born animal contains not only a smaller amount but also a smaller percentage of calcium than does the body of a normal adult of the same species.

Von Liebig's generalization grew mainly out of his observations upon the growth of plants, especially when supplied with salt mixtures in which some one essential element was lacking or so reduced in amount as to become the growth-limiting factor.

Osborne and Mendel (1) performed similar experiments with animals, feeding them with mixtures of purified organic and inorganic foodstuffs, so devised as to permit the study of the effect of an almost complete deprivation of any desired element. Young rats thus subjected to drastic deprivation of calcium intake showed an early cessation of growth.

When Aron and Sebaauer (2) had fed young dogs on mixtures of cracker-meal and meat with or without bone, the calcium-poor ration being by no means as completely devoid of this element as were those of Osborne and Mendel, the shortage of calcium usually but not always resulted in stunting the growth. Of two animals which they contrast in detail, one received bone with his food and developed normally, the other while on the calcium-poor diet (without bone) made an equal gain in weight but his bones lacked normal rigidity and on analysis were found to be low in calcium and total mineral content and unduly watery in their composition.

Sherman and MacLeod (3) made a systematic series of determinations of calcium in normal white rats of known nutritional history which were killed for analysis at definite age intervals, thus establishing quantitatively a scale of normal percentages of body calcium for such rats at different ages. Together with these data for normal animals, they reported a number of analyses of rats which had been more or less stunted in growth by different dietary deficiencies. These showed that the bodies of the young animals had a strong tendency to increase their percentage of calcium with time (age) even when growth in the usual sense of increase in body mass was inhibited. With calcium intake considerably greater than in the experiments of Osborne and Mendel mentioned above, but considerably less than that supplied in the diets regarded as fully adequate, there was increase in body mass and in body calcium; but, as the amount of calcium furnished by the food did not support a rate of calcification equal to that in the

animals with higher calcium intake, the animals which had received calcium-poor food were found to have more or less calcium-poor bodies as compared with those of the same age which were regarded as normal.

The investigation here reported was especially designed to elucidate further the relationship between the calcium content of the body and that of the food, or the influence of graded intakes of calcium upon the rate of increase of the percentage of calcium in the growing body.

EXPERIMENTAL

White rats of the same strain, and each of known nutritional history, were raised to definite ages on diets varying in calcium content and the entire bodies analyzed for calcium. The calcium content of the diets for one series of animals was varied by varying the proportion of two natural foods, wheat and milk, in the food mixture; for a second series of animals, food mixtures similar to these with respect to their calcium content were employed which contained enough natural food to insure against deficiency of any still unknown growth essential, together with artificially purified nutrients in such proportions as to make calcium the sole significant variable of the food. The animals were killed with chloroform and the bodies incinerated in silica dishes. The fact having previously been established that the walls of the digestive tract of the rat contain so small an amount of calcium as to be wholly negligible as compared with the total body calcium with which we are here concerned, the digestive tract together with its contents was rejected in the preparation of the bodies of the experimental animals for analysis. After charring over an open flame, the bodies of the animals were ashed in a muffle furnace regulated at dull red heat. The ash was dissolved in hydrochloric acid, filtered, and diluted to a suitable volume. Aliquot portions of the solution were used for the quantitative determination of calcium according to the method of McCrudden. The percentage of calcium in the body was calculated on the basis of the net body weight; that is, the last live weight minus the weight of the contents of the digestive tract.

First Series

In this series the calcium content of the diet was varied by varying the proportions of the two natural foods, wheat and milk, in the food mixture. Five diets containing different proportions of wheat and milk were fed. In each case sodium chloride was added to the diet in amounts equal to 2 per cent of the weight of the wheat. The proportions of milk powder and wheat in the diets were by weight, as follows: 1 part of milk powder to 9 parts of wheat, 1 part of milk powder to 5 parts of wheat, 1 part of milk powder to 2 parts of wheat, 1 part of milk powder to 1 part of wheat, and 2 parts of milk powder to 1 part of wheat. The calcium and phosphorus contents of these diets were respectively: 0.13 per cent calcium, 0.38 per cent phosphorus; 0.19 per cent calcium, 0.44 per cent phosphorus; 0.33 per cent calcium, 0.51 per cent phosphorus; 0.48 per cent calcium, 0.56 per cent phosphorus; and 0.63 per cent calcium, 0.63 per cent phosphorus.

Animals raised on these different diets were analyzed for calcium at 28 to 30 days, 60 days, 90 days, 120 days, and 180 to 220 days of age. From 220 days on, animals were not analyzed at definite age intervals but were collectively considered as adults.

The second and third of the diets described above, containing respectively 0.19 per cent and 0.33 per cent of calcium were also in use in our colony for numerous families of rats throughout successive generations. By analysis of the young from such families it was possible to include, in the present study, a comparison of the calcium content of new born, 15 day, and 30 day old rats from families of the same stock but whose nutritional history differed as just stated.

The average calcium content of new born rats of families on the diets of milk and wheat in which the percentages of calcium were 0.19 and 0.33, was 12.4 mg. or 0.28 per cent and 12.1 mg. or 0.28 per cent, respectively. The average calcium content of new born rats of various other nutritional histories was 12.0 mg. or 0.26 per cent. In view of the limitations of precision in the measurement of these small quantities of calcium, the influence of nutritional history upon the calcium content of new born animals would seem not to be definitely determinable until larger animals are employed.

At 15 days of age, the young rats of the families which were fed on the milk and wheat diet containing 0.33 per cent calcium had

deposited approximately 50 per cent more body calcium than those of the families nourished on the diet containing 0.19 per cent calcium.

In the case of the 15 day old rats, as well as those of each greater age on the milk and wheat diets, the average body weights increased somewhat with increase of the calcium content of the food. However, with increase in the calcium content of these diets, due to the increase in proportion of milk, the diets were also improved with respect to certain other essential food factors as well.

The data of this first series of experiments are summarized in Table I and will be discussed below, jointly with the data of the experiments of the second series.

TABLE I
Average Calcium Content of Rats at Different Ages

Sex	Ca content of diet	No. of animals	Average body weight	Ca in body	
New born rats					
	<i>per cent</i>		<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
	0.19	11	4.3	0.0124 \pm 0.0021†	0.28 \pm 0.03‡
	0.33*	17	4.4	0.0121 \pm 0.0014	0.28 \pm 0.04
	Miscellaneous†	9	4.6	0.0120 \pm 0.0017	0.26 \pm 0.01
15 day old rats					
M.	0.19	5	14.8	0.0953 \pm 0.0167	0.65 \pm 0.03
"	0.33*	9	22.5	0.1342 \pm 0.0100	0.60 \pm 0.03
F.	0.19	5	13.1	0.0862 \pm 0.0143	0.67 \pm 0.05
"	0.33*	10	22.3	0.1364 \pm 0.0058	0.62 \pm 0.04
28 to 30 day old rats					
M.	0.19	6	31.6	0.2400 \pm 0.0138	0.76 \pm 0.02
"	0.33*	22	49.5	0.3317 \pm 0.0490	0.67 \pm 0.04
"	0.63	8	46.2	0.3524 \pm 0.0825	0.76 \pm 0.04
F.	0.13	3	15.0	0.1281 \pm 0.0028	0.84 \pm 0.04
"	0.19	10	36.4	0.2833 \pm 0.0489	0.79 \pm 0.03
"	0.33*	16	40.5	0.3007 \pm 0.0366	0.74 \pm 0.04
"	0.63	6	40.8	0.3255 \pm 0.0768	0.79 \pm 0.03

* Includes analyses by Sherman and MacLeod.

† The preceding generations were on miscellaneous diets.

‡ The figures in this column express average deviation.

TABLE I—*Concluded*

Sex	Ca content of diet	No of animals	Average body weight	Average net weight	Ca in body		
60 day old rats							
	<i>per cent</i>		<i>gm.</i>	<i>gm.</i>	<i>gm</i>	<i>per cent</i>	
M.	0.19*	7	117	113	0.707 ± 0.077†	0.63 ± 0.02†	
"	0.33*	11	138	135	1.023 ± 0.156	0.76 ± 0.02	
"	0.48	2	128	123	1.131 ± 0.001	0.94 ± 0.03	
"	0.63	4	150	143	1.268 ± 0.191	0.89 ± 0.06	
F.	0.19*	6	110	105	0.716 ± 0.060	0.68 ± 0.03	
"	0.33*	8	111	107	0.914 ± 0.133	0.86 ± 0.02	
"	0.48	2	115	111	1.081 ± 0.032	0.98 ± 0.02	
"	0.63	3	134	128	1.213 ± 0.108	0.95 ± 0.02	
90 day old rats							
M.	0.19*	11	163	157	1.109 ± 0.198	0.70 ± 0.03	
"	0.33*	13	222	217	2.013 ± 0.135	0.93 ± 0.04	
F.	0.19*	9	135	130	1.050 ± 0.153	0.80 ± 0.03	
"	0.33*	11	161	157	1.712 ± 0.140	1.09 ± 0.03	
"	0.63	4	175	187	1.849 ± 0.115	1.11 ± 0.03	
120 day old rats							
M.	0.33*	8	258	252	2.517 ± 0.231	1.01 ± 0.03	
F.	0.33*	8	193	188	1.998 ± 0.122	1.07 ± 0.06	
"	0.63	4	193	188	2.291 ± 0.182	1.20 ± 0.05	
Sex	Ca content of diet	No of animals	Average maximum body weight	Average final weight	Average net final weight	Ca in body	
180 to 220 day old rats							
	<i>per cent</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>	
M.	0.13	2	114	114	112	1.076 ± 0.520	0.82 ± 0.12
"	0.19	5	236	232	220	2.179 ± 0.257	0.98 ± 0.05
"	0.33*	3	303	299	294	3.125 ± 0.526	1.06 ± 0.02
"	0.48	3	310	309	303	3.120 ± 0.201	1.03 ± 0.03
F.	0.13	2	105	105	105	0.847 ± 0.069	0.84 ± 0.01
"	0.19	5	146	144	137	1.532 ± 0.117	1.12 ± 0.03
"	0.33*	3	213	212	208	2.676 ± 0.426	1.13 ± 0.06

* Includes analyses by Sherman and MacLeod.

† The figures in this column express average deviation.

Second Series

In these experiments upon the relation of the calcium content of the body to that of the food when calcium is the sole significant

TABLE II
Average Calcium Content of Rats on Diets in Which Calcium Was the Sole Significant Variable

Age	Sex	Ca content of diet	No of rats	Average body weight	Average net body weight	Ca in body	
<i>days</i>		<i>per cent</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
60	M.	0.16	2	114	108	$0.746 \pm 0.106^*$	$0.70 \pm 0.03^*$
	"	0.21	3	137	127	0.860 ± 0.057	0.68 ± 0.04
	"	0.32	2	127	118	1.007 ± 0.012	0.87 ± 0.10
	"	0.50	1	94	89	0.853	0.96
	F.	0.16	3	95	87	0.637 ± 0.088	0.76 ± 0.08
	"	0.21	4	100	93	0.763 ± 0.040	0.83 ± 0.05
	"	0.32	4	105	97	0.889 ± 0.089	0.92 ± 0.05
	"	0.50	5	97	90	0.922 ± 0.166	1.03 ± 0.06
90	M.	0.16	3	206	191	1.257 ± 0.090	0.66 ± 0.01
	"	0.21	3	233	221	1.602 ± 0.189	0.75 ± 0.09
	"	0.32	2	155	142	1.368 ± 0.154	0.98 ± 0.05
	"	0.50	3	204	194	1.799 ± 0.193	0.92 ± 0.02
	F.	0.16	3	156	144	1.116 ± 0.018	0.78 ± 0.04
	"	0.21	3	124	118	1.136 ± 0.067	0.97 ± 0.03
	"	0.32	2	131	124	1.315 ± 0.166	1.06 ± 0.01
	"	0.50	3	177	168	1.780 ± 0.036	1.06 ± 0.02
180	M.	0.16	2	274	263	2.366 ± 0.064	0.90 ± 0.01
	"	0.21	3	321	308	2.784 ± 0.046	0.91 ± 0.02
	"	0.32	3	318	299	2.954 ± 0.142	0.99 ± 0.04
	"	0.50	3	280	270	2.792 ± 0.068	1.04 ± 0.02
	F.	0.16	1	199	190	1.946	1.02
	"	0.21	2	181	173	1.954 ± 0.043	1.13 ± 0.01
	"	0.32	3	209	198	2.304 ± 0.054	1.16 ± 0.03
	"	0.50	2	203	190	2.317 ± 0.033	1.22 ± 0.02

*The figures in this column express average deviation.

variable of the diet, four food mixtures containin respectively 0.16, 0.21, 0.32, and 0.50 per cent of calcium were fed.

That of lowest calcium content contained per 1,000 gm. of diet as follows: 100 gm. of whole milk powder, 55 gm. of casein, 68

gm. of butter fat, 16 gm. of sodium chloride, and 777 gm. of ground whole wheat. This diet was found by analysis to contain 0.16 per cent calcium and 0.42 per cent phosphorus.

Diets of higher calcium contents were made by substituting in this diet 3.3, 11.7, and 25.8 gm. of calcium lactate per 1,000 gm. of diet for equal weights of wheat. The respective calcium contents of these diets were 0.21, 0.32, and 0.50 per cent, the phosphorus content remaining constant at 0.42 per cent. The ratio of the substituted materials to the entire food mixture was so small in each case that the energy value and composition of the food mixture, other than its calcium content, were not significantly affected.

Animals whose preceding generations had been reared on the milk and wheat diet, of which the calcium content was 0.33 per cent, were used for these experiments. At the age of 28 days the rats were separated from the mothers and placed in individual cages. A part of each litter was raised on each of the four experimental diets. At the ages of 60, 90, and 180 days, animals on each of the diets were killed and analyzed for calcium as already described.

The growth in body weight was practically the same on all four of the diets used in this series. The average total body calcium and the average percentage of calcium in the bodies of the males and females, separately considered, and on any one of the four diets increased continuously with increase of age. For the same sex at each of the definite ages, the total body calcium and the percentages of calcium in the body increased progressively with increase in the calcium content of the food. The results of this series as regards growth in body weight and calcium content of the body are given in Table II.

Discussion and Summary of Results

Both when the diet was composed of natural food materials and when it was so arranged as to make calcium the sole significant variable factor, it was found that at each of the ages studied in this investigation the amount of calcium in the body of the growing animal varied in accordance with the calcium content of its food.

Among the rats fed calcium-poor diets of natural food materials, there was at the early ages of 15 and 28 to 30 days a tendency

to increase so slowly in body weight that, while the total amount of body calcium varied in accordance with the calcium intake, the percentage of body calcium was higher for those animals whose growth rate was distinctly inferior to that of animals on the better diets.

In both series of animals the percentages of calcium in the bodies of the rats of either sex from 60 to 180 days of age increased with increase in the calcium content of the food. The essential characteristics of this relationship are graphically represented, for the female animals of the second series, in Fig. 1.

Those experimental animals which grew to middle-aged adult

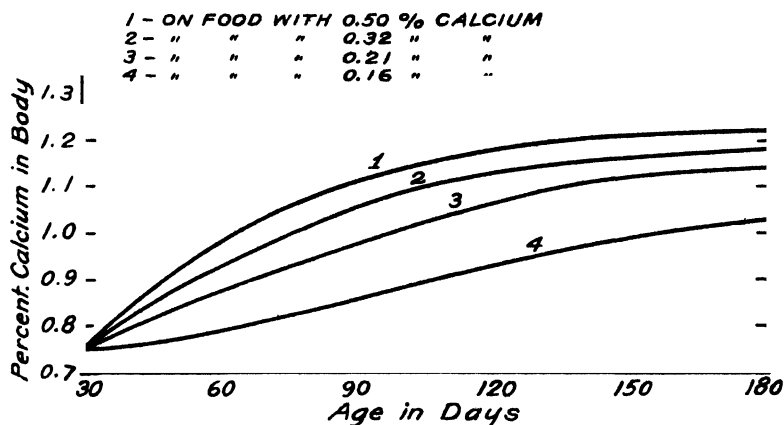


FIG. 1. Percentage of calcium in the body in relation to age and to the calcium content of the food (smoothed curves for females of second series).

life and which may reasonably be considered normal adults as shown by the fact that they had successfully reproduced themselves generation after generation, in spite of being reared on diets quite different with respect to calcium content, here reached approximately a common value for percentage of body calcium. The average of the percentages of calcium in the bodies of normal adult male rats was 1.09 per cent and of normal adult female rats 1.23 per cent. These facts would indicate that the animal body is able to retain calcium at a subnormal rate on relatively calcium-poor diets and so at an abnormally late age may reach the percentage of body calcium of normal middle-aged adult animals.

As may be seen from the Tables I and II, or more readily from Fig. 1, the animals receiving the more liberal amounts of calcium here fed (0.48 or 0.50 per cent of the air-dry food mixture) had at 6 to 7 months of age practically reached the average percentage of body calcium of normal adults; but those whose food contained progressively less calcium had bodies progressively poorer in calcium even at this age at which growth in size was very nearly complete.

As previously noted, the animals of the second series, in whose food calcium was the sole significant variable, grew at practically the same rate on each of the four levels of calcium intake. Moreover, those of them receiving the lower calcium intakes had every physical appearance of being as well nourished as those reared on the diet richest in calcium; but chemical analysis showed that their bodies were calcium-poor.

While von Liebig's "law of the minimum" may not often be quoted by name, the point of view which it formulated has now become so much a part of our habit of thought, that when we see what appears to be normal growth we are apt to assume that this means actually normal development. And probably this is generally true; but it is important to note that the calcium relationships here discussed must be regarded either as an exception to this generalization or, perhaps better, as requiring a less simple and more discriminating application of the principle.

The significance of this fact is more fully apparent when considered further in the light of the effects of the calcium intake upon the rate of calcium retention in growing children as determined by Sherman and Hawley (4), who found that while calcium intakes of about 0.4 gm. per day regularly resulted in some retention of this element, it was necessary to feed from 0.9 to 1.2 gm. per day in order to induce optimal calcium retention. The children receiving the higher amounts of calcium and making the higher rate of development with respect to body calcium are in a condition analogous to that of the rats of the uppermost curve of Fig. 1; those on lower calcium intakes, analogous to the rats represented by the lower curves, stored calcium but at a subnormal rate so that they were retarded in reaching the truly normal percentage of body calcium for their age.

By taking account both of the calcium balance experiments just

mentioned, and of the determinations of body calcium by analysis of experimental animals as here reported, it is plain that the calcium-poor condition of body in children may coexist throughout much or all of the period of growth with normal increases of height and weight and with every appearance of good health as indicated by physical examination.

Until the body has attained the normal percentage of calcium for its age, we consider the maximal rate of calcium retention is the optimal.

This is true whether we think of the increasing calcium content of the bones simply as a matter of their attainment of their normal adult structure, or whether, with Bauer, Aub, and Albright (5), we regard a part of the bone calcium as in some sense constituting a bodily reserve of this element.

In an experiment extending through several generations (6), and covering large numbers of rats of both sexes, it has been found that the diets of higher calcium content which permit more rapid calcification of the growing body result not only in somewhat earlier maturity but also and in greater measure they extend the period which lies between the attainment of maturity and the appearance of senescence.

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THE BASIC AMINO ACIDS OF SILK FIBROIN. THE DETERMINATION OF THE BASIC AMINO ACIDS YIELDED BY PROTEINS*

BY HUBERT BRADFORD VICKERY AND RICHARD J. BLOCK

(From the Biochemical Laboratory of the Connecticut Agricultural Experiment Station, New Haven, and the Laboratory of Physiological Chemistry, Yale University, New Haven)

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Previous investigations of the basic amino acids yielded by human hair and by wool (1, 2) showed that the keratins of these closely allied tissues yield relatively high proportions of arginine but very low proportions of histidine; both tissues also yield high proportions of cystine. Because of this the determination of the basic amino acids presented special analytical difficulties that necessitated slight modifications in the technique employed. Available information regarding the composition of silk fibroin suggested that the unusually high proportion of monoamino acids, that results from the hydrolysis of this material, might also give rise to analytical difficulties, a study of which would be useful in the development of the methods of protein analysis. This was indeed found to be the case and the further interesting observation was made that the ratios of the yields of the three basic amino acids from silk fibroin are very closely similar to those from wool, although the *total* yield of bases from silk fibroin is only about one-tenth of that from wool.

Very little attention has been paid in the past to the basic amino acids derived from silk. Wetzel, in 1899 (3), obtained a trace of histidine, and Fischer and Skita, in 1902 (4), isolated somewhat under 1 per cent of arginine. Abderhalden, in 1922 (5),

* The data in this paper are taken from the dissertation submitted by R. J. Block in partial fulfilment of the requirement for the degree of Doctor of Philosophy, Yale University, 1931. A part of the expense of this investigation was borne by the Carnegie Institution of Washington, D. C.

found 1.5 per cent of arginine, 0.75 per cent of histidine, and 0.85 per cent of lysine by the Kossel and Kutscher (6) method; all three bases were secured as crystalline derivatives, but the proportions reported were probably calculated from the nitrogen content of their respective fractions, rather than from the weights of the derivatives, and the results may therefore be somewhat too high. Fürth and Deutschberger (7) obtained 1.52 per cent of arginine as the flavianate, but neglected to establish the purity of their product by analysis. It is clear, therefore, that the composition of silk fibroin, with respect to the basic amino acids, is still uncertain.

First Analysis—The silk fibroin employed contained 18.99 per cent of nitrogen, corrected for moisture and ash, and was free from sulfur. The sericin had been removed commercially;¹ 439 gm. (corrected) were boiled with 3800 cc. of 8 N sulfuric acid for 16 hours; the hydrolysate was then diluted, neutralized to pH 4 with barium hydroxide, and the barium sulfate was removed and extensively washed. The hydrolysate was concentrated to 5 liters and silver oxide and dilute sulfuric acid were added alternately, according to Kiesel's (8) directions, until silver sulfate crystallized in considerable amounts from the warm solution. In spite of this the customary test for the presence of excess silver ion could not be obtained. Barium hydroxide was nevertheless added until a reaction of approximately pH 12 had been produced and the precipitate was removed. The filtrate was acidified and concentrated, and a second unsuccessful attempt to introduce an excess of silver ion was made. The precipitate, produced by making the solution alkaline with barium hydroxide, was added to the first precipitate and the material was worked up for histidine and arginine as described by Vickery and Block (2). The equivalents of 0.076 per cent of histidine and 0.22 per cent of arginine were isolated as flavianates.

The combined filtrates from the two silver precipitates were freed from barium and silver and treated with phosphotungstic acid in the usual way. In view of the low proportion of arginine found in the silver precipitate the phosphotungstate precipitate was investigated for arginine. No difficulty was experienced in

¹ We are indebted to Dr. E. M. Shelton of Manchester, Connecticut, for this highly purified material.

introducing excess of silver ion into the acidified solution of the bases by the silver oxide technique, and the precipitate obtained at alkaline reaction yielded the equivalent of 0.52 per cent more arginine as the flavianate. No histidine could be detected. The filtrate from this silver precipitation was freed from reagents and treated again with phosphotungstic acid. Lysine was isolated from it, as picrate of decomposition point 265° , in an amount equivalent to 0.25 per cent of the fibroin.

It is evident therefore that, in order to precipitate arginine completely, it is essential to introduce a liberal excess of silver ion into the solution of the amino acids before this is made alkaline. Histidine, however, is completely precipitated even when silver ion is not present in excess as shown by the customary test.

Repeated experiences similar to that described have convinced us that silver sulfate, in spite of its manifest convenience, is not a suitable reagent to employ for the *preliminary* precipitation of arginine and histidine from protein hydrolysates.

A number of factors appear to affect the concentration of the silver ion that can be produced; among these are the acidity, the nature, and the concentration of the amino acids in the solution. There seems little doubt that silver enters into complex compounds with the amino acids but a satisfactory explanation of the phenomena observed has not yet been obtained.

Second Analysis—In view of the foregoing observations a hydrolysate of 440 gm. (corrected) of silk fibroin was prepared as before and was treated, at a volume of 12 liters, with a saturated solution of silver nitrate; 500 gm. of the salt were added before a strongly positive test for the presence of excess silver ion was secured. The solution was then made alkaline as usual; the precipitate produced yielded the equivalent of 0.7 per cent of arginine, as the monoflavianate of sulfur content 6.8 per cent (theory 6.56 per cent), and 0.06 per cent of histidine, as the diflavianate of sulfur content 8.13 per cent (theory 8.17 per cent). The filtrate from the silver precipitate was not investigated for lysine.

The results of these analyses are given in Table I together with previous analyses of silk collected from the literature; the proportions of bases isolated from wool are appended in order to draw attention to the close similarity in the ratios of the three bases to each other in these two proteins. The discrepancy between the

present analyses and those of others is probably largely due to a difference in method. The isolation method we have used leads to minimal values, while the calculation of results from nitrogen determinations leads to maximal values. It is probable that the true value is intermediate. It is also not improbable that silk of different origins may be differently constituted; this point should be further investigated.

Estimation of the Basic Amino Acids Yielded by Proteins

In the course of the studies by Vickery and Leavenworth of the silver precipitation method for the determination of the basic amino acids, a number of modifications and changes have been introduced from time to time that are described in detail in differ-

TABLE I
Basic Amino Acids Yielded by Silk Fibroin

	Silk fibroin				Wool
	Analysis I	Analysis II	Abderhalden (5)	Fischer and Skita (4)	Vickery and Block (2)
	per cent	per cent	per cent	per cent	per cent
Arginine.....	0.74	0.7	1.5	1.0	7.8
Histidine.....	0.076	0.06	0.75		0.7
Lysine.....	0.25		0.85		2.3

ent papers (9-11, 1). It therefore seems desirable to give a brief description of what now appears to be the most generally applicable procedure. Unless otherwise noted it is to be understood that all precipitates are washed and all concentrations are carried out *in vacuo*. The volumes at which it is suggested that the different precipitations should be conducted are suitable in the case of proteins, such as edestin, that yield somewhat high proportions of bases; smaller volumes should be used where conditions warrant.

Hydrolysis—At least 50 gm. of protein are hydrolyzed by boiling for 24 or more hours with 8 times its weight of 8 N sulfuric acid. The hydrolysate is diluted and the greater part of the acid is removed as barium sulfate. As an alternative procedure the protein is boiled with 8 times its weight of 6 N hydrochloric acid,

the solution is repeatedly evaporated to a sirup, this is diluted, and the hydrochloric acid is removed as silver chloride by means of silver oxide in the presence of sufficient sulfuric acid to maintain the reaction somewhat more acid than pH 3. The silver chloride should be suspended in hot water, acidified with hydrochloric acid, and digested on the steam bath in order to recover any histidine that may have been precipitated. The extract is evaporated to dryness, the residue is taken up in water, and the hydrochloric acid is removed as before, the filtrate from the silver chloride being added to the main solution.

Silver Precipitation—The hydrolysate and washings of the inorganic precipitate are concentrated to a volume of 1 liter for each 50 gm. of protein. Silver nitrate is added, in concentrated solution, until the test for an excess of silver ion is strongly positive. Warm saturated barium hydroxide is then added until the solution is alkaline to alizarin yellow R (pH 11 to 12), the precipitate is centrifuged off and, without washing, is suspended in water and decomposed by hydrogen sulfide in the presence of a slight excess of sulfuric acid (pH 3 to 4). After removal of the silver sulfide and concentration to the same volume as before, silver oxide and sulfuric acid are added alternately until an excess of silver ion is present. The reaction is then brought to pH 7.2 by the cautious addition of cold saturated barium hydroxide solution (for details see (12), p. 119), the precipitate is centrifuged off and, without washing, is decomposed as before by hydrogen sulfide, in the presence of sulfuric acid. The solution is concentrated to about half the previous volume and the precipitation with silver at pH 7.2 is repeated (9). The precipitate is centrifuged and, if nitrate ion is found in the filtrate, must be washed until this can no longer be detected. It is then suspended in water, acidified to *litmus* with sulfuric acid, and decomposed with hydrogen sulfide; the solution is the crude histidine fraction. After concentration the reaction should be adjusted with barium hydroxide to approximately pH 4.

In order to remove the cystine that is precipitated along with the histidine (1), dry copper hydroxide (Kahlbaum), or dry copper carbonate, is added in excess to the boiling solution at a volume of approximately 250 cc. for each 50 gm. of protein. The solution is then allowed to digest on the steam bath for an hour, with

occasional stirring, and is finally chilled overnight. The filtrate from the precipitate is freed from copper by hydrogen sulfide, is concentrated, and the histidine is precipitated by mercuric sulfate in the presence of 5 per cent by weight of sulfuric acid (for details see (10), p. 709), and finally isolated as the diflavinate.

Isolation of Arginine—The combined filtrates from the two precipitates at pH 7.2 are acidified to pH 3 to 4 with sulfuric acid and concentrated to a volume of 1 liter for each 50 gm. of protein; if excess of silver ion is not present sufficient silver oxide is added to provide this and the solution is then brought to pH 11 to 12 with warm saturated barium hydroxide. The precipitate is washed free from nitrate ion, if this is present, by disintegrating and centrifuging with very dilute barium hydroxide solution (twice is usually sufficient), is suspended in water, acidified to litmus with sulfuric acid, and is then decomposed with hydrogen sulfide. The solution, after concentration, should be faintly acid to Congo red (pH 4 to 5) and must be free from barium. It is brought to a definite volume and the nitrogen content is determined. Arginine is isolated as the monoflavinate from a suitable aliquot part (for details see (10), p. 709).

Isolation of Lysine—The filtrates from the two silver precipitates at alkaline reaction are combined, acidified with sulfuric acid, and the solution is saturated with hydrogen sulfide, or, as an alternative procedure, is treated with an excess of barium sulfide. After making certain that the barium has been completely precipitated the barium sulfate and silver sulfide are removed. The solution is concentrated slightly to remove hydrogen sulfide and neutralized to Congo red with sodium hydroxide; it is then further concentrated to a volume of about 500 cc. for each 50 gm. of protein. The solution is made alkaline with sodium hydroxide, an equal volume of alcohol is added, and the solution is again concentrated *in vacuo* at as low a temperature as possible to remove ammonia. Great care must be exercised at this point; as little excess sodium hydroxide as possible should be used. The concentrated solution is acidified with sulfuric acid, is filtered if necessary, and brought to a volume of 500 cc. for each 50 gm. of protein; sufficient sulfuric acid is added to make a concentration of 5 per cent by weight and the lysine is precipitated by the addition of an excess of phosphotungstic acid. The precipitate is

removed, after standing overnight, and, without washing, is dissolved in 50 per cent acetone and decomposed by barium hydroxide. The barium phosphotungstate is thoroughly washed with warm and finally with hot water made alkaline with barium hydroxide, the solution is acidified with sulfuric acid, is concentrated to remove all acetone, and the lysine is precipitated a second time as phosphotungstate in the presence of 5 per cent of sulfuric acid. The precipitate is washed free from nitrate ion by disintegrating and centrifuging with a 2.5 per cent solution of phosphotungstic acid in 5 per cent sulfuric acid (three times are usually sufficient). After decomposition in acetone solution with recrystallized barium hydroxide the lysine is isolated as the picrate (for details see (10), p. 710). The double precipitation of lysine with phosphotungstic acid accomplishes a very considerable purification of the lysine fraction with respect to other amino acids as well as a complete elimination of inorganic ions.

SUMMARY

If silver sulfate is employed during the precipitation of arginine and histidine from protein hydrolysates that contain an unusually high proportion of monoamino acids, difficulty is experienced in introducing an adequate excess of silver ion; moreover, unless a considerable excess of silver ion is present a large part of the arginine may escape precipitation. These difficulties are avoided if silver nitrate is employed for the preliminary precipitation. Silver sulfate is then used for the reprecipitation and separation of the two bases and the laborious washing that is required to remove nitric acid from the precipitates is thereby largely avoided.

Silk fibroin has been found to yield 0.74 per cent of arginine, 0.07 per cent of histidine, and 0.25 per cent of lysine. A close analogy between the relative proportions of the bases yielded by silk fibroin and by wool has been noted.

A brief description of the most widely applicable method for the determination of the basic amino acids in proteins is given. .

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THE BASIC AMINO ACIDS OF PROTEINS. A CHEMICAL RELATIONSHIP BETWEEN VARIOUS KERATINS*

BY RICHARD J. BLOCK AND HUBERT BRADFORD VICKERY

(From the Laboratory of Physiological Chemistry, Yale University, New Haven, and the Biochemical Laboratory of the Connecticut Agricultural Experiment Station, New Haven)

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A classification of proteins on the basis of their chemical composition rather than of their solubility has long been desired, but, in spite of the numerous investigations of the amino acid composition of these substances by Fischer, Abderhalden, Osborne, and others, no *general* grouping, founded on the proportions of the different amino acids which they yield, has yet been proposed. The protamines, however, have been divided by Kossel (1) into groups according to the nature and relative proportion of the basic amino acids found among the products of their hydrolysis, and the investigations of the mucoproteins by Levene (2) have shown that these conjugated proteins can also be classified according to the nature of the so called prosthetic group.

Our studies of a number of the highly insoluble proteins usually grouped together as keratins, have shown that these substances or, rather, tissues, yield markedly similar relative proportions of the basic amino acids after complete hydrolysis. It therefore seems possible that a tentative classification of the keratins may be founded upon this result. That a marked similarity exists in the amino acid make-up of some of them has already been pointed out by Buchtala (3), but definite experimental support for an attempted classification has not hitherto been obtained.

Our analyses were conducted by the silver precipitation method,

* The data in this paper are taken from the dissertation submitted by R. J. Block in partial fulfilment of the requirement for the degree of Doctor of Philosophy, Yale University, 1931. A part of the expense of this investigation was borne by the Carnegie Institution of Washington, D. C.

for the most part according to the modifications introduced by Vickery and Leavenworth (4, 5). Several additional changes in detail were introduced during the progress of the work; these have been described by Vickery and Block (6).

*A. Basic Amino Acids from the Corals, Gorgonia flabellum and Plexaurella dichotoma*¹

Krukenberg, many years ago (7), suggested that the horny axial skeleton of Gorgonian corals is related to the albuminoids of the higher animals because it contains nitrogen and is not digested by gastric nor by pancreatic juice. Drechsel (8) substantiated this idea when he showed that gorgonin, from the Mediterranean species, *Gorgonia cavolinii*, yielded ammonia and hydrogen sulfide on burning, and lysine, tyrosine, "leucine," and "lysatinin" on appropriate treatment of an acid hydrolysate. Henze, in 1903 (9), obtained approximately 4 per cent of arginine as the nitrate, less than 3 per cent of lysine as the picrate, and a trace of histidine dihydrochloride from 50 gm. of gorgonin from the same species. Kossel and Kutscher (10) reported 5 per cent of arginine and 3 per cent of lysine obtained from the closely analogous material spongin, the skeleton of the common sponge. They did not succeed, however, in detecting histidine.

A specimen of the Florida species *Gorgonia flabellum* was ground, decalcified for 2 weeks with dilute hydrochloric acid, and was then washed and dried. The residue contained 15.6 per cent of nitrogen, 1.1 per cent of sulfur, and 1.4 per cent of ash; 41.8 gm. of the material (corrected for moisture and ash) were hydrolyzed with hydrochloric acid. Arginine was isolated as the flavianate in an amount equivalent to 6.4 per cent; when corrected for the solubility of arginine silver in 3.5 liters of solution this was equal to 7.3 per cent of arginine. The arginine flavianate contained 6.6 per cent of sulfur, theory 6.56 per cent. Histidine was isolated as the diflavianate equivalent to 0.48 per cent; the preparation contained 8.25 per cent of sulfur, theory 8.17 per cent. Lysine was isolated as the picrate of decomposition point 262° in an amount equivalent to 2.75 per cent of the protein.

¹ We wish to express our thanks to Professor Lafayette B. Mendel, and to Dr. W. H. Longley of the Tortugas Laboratory of the Carnegie Institution of Washington, for the specimens used in these analyses.

Plexaurella dichotoma is closely related to *Gorgonia flabellum*. The keratin was prepared in essentially the same way and contained 15.6 per cent of nitrogen, 1.73 per cent of sulfur, and 2.0 per cent of ash. From the 14.3 gm. (corrected for moisture and ash) hydrolyzed, we obtained 5.4 per cent of arginine as flavianate (sulfur content 6.61 per cent), 0.43 per cent of histidine as diflavianate (sulfur content 8.2 per cent), and somewhat less than 3 per cent of lysine. The crude lysine picrate decomposed at 250° but, owing to an accidental loss, only a small amount of pure lysine picrate (melting point 263°) was obtained.

It is evident that the skeletons of the American corals, *Gorgonia flabellum* and *Plexaurella dichotoma*, yield proportions of the basic amino acids not unlike those obtained from gorgonin by Henze and from spongin by Kossel and Kutscher.

B. Basic Amino Acids from Goose Feathers

No previous determination of the basic amino acids yielded by feathers was found in the literature. The clean, dry, fat-free feathers contained 15.5 per cent of nitrogen and 3.2 per cent of sulfur. The basic amino acids were isolated from 167 gm. (corrected for moisture and ash) of material. Arginine flavianate, of sulfur content 6.7 per cent, equivalent to 4.8 per cent of arginine was obtained; corrected for the solubility of arginine silver in 12 liters of solution, this was equivalent to 5.0 per cent of arginine. Histidine diflavianate of sulfur content 8.24 per cent equivalent to 0.35 per cent of histidine, and lysine picrate of decomposition point 264° equivalent to 1.04 per cent of lysine were also isolated.

C. Basic Amino Acids from Snake Epidermis²

The skins (python and cobra) were comminuted and thoroughly extracted with hot water, very dilute hydrochloric acid, absolute alcohol, and ether. The dried material contained 17.1 per cent of nitrogen and 2.6 per cent of sulfur corrected for moisture and ash. Of this, 83.1 gm. were hydrolyzed with sulfuric acid, and 6.1 per cent of arginine as flavianate, of sulfur content 6.58 per cent, was isolated. Histidine was isolated as the diflavianate, of sulfur

² The material used in these experiments was obtained through the kindness of Dr. Raymond L. Ditmars of the Bronx Zoological Gardens, New York City.

content 8.17 per cent, equivalent to 0.45 per cent, and lysine as the picrate of decomposition point 263° equivalent to 1.4 per cent.

TABLE I
Basic Amino Acids of Various Keratins

Protein	Arginine		Histidine isolated as di-flavinate	Lysine isolated as picrate	Cystine, colorimetric determination
	Isolated as flavinate	Corrected for solubility			
	per cent	per cent	per cent	per cent	per cent
Human hair (5)	8.0		0.5	2.5	16.5
Sheep wool (11).....	7.8	8.6	0.66	2.3	10.0
Snake epidermis.....	5.4	6.1	0.48	1.4	5.3
Goose feathers.....	4.8	5.0	0.35	1.04	6.4
<i>Gorgonia flabellum</i>	6.4	7.3	0.48	2.75	5.5
<i>Plezaurella dichotoma</i>	5.4	6.6	0.43	3.00*	3.2
Silk fibroin.....	0.74		0.077	0.25	0.0

* This figure is based on the weight of the crude picrate; most of the specimen was accidentally lost during recrystallization.

TABLE II
Molecular Ratios of the Basic Amino Acids

	Arginine : histidine	Arginine : lysine	Lysine : histidine
Human hair..	14	3.0	5.0
Sheep wool.	11	2.9	3.7
Snake epidermis	12	3.6	3.3
Goose feathers..	14	3.9	3.6
<i>Gorgonia flabellum</i>	13	2.0	6.6
<i>Plezaurella dichotoma</i>	12		
Silk fibroin.....	9	2.5	3.6
Average	12	3	4.3

CONCLUSION

A summary of the proportions of the basic amino acids yielded by the keratins that have been analyzed by the modified silver precipitation technique is given in Table I and a calculation of the molecular ratios of the bases to each other is given in Table II.

Over the entire series of keratins the average ratio of the histidine:lysine:arginine is not far from 1:4:12. The fact that the keratins of human hair, sheep wool, goose feathers, snake epidermis, *Plexaurella dichotoma*, *Gorgonia flabellum*, and silk fibroin, obtained from animals widely separated phylogenetically, should yield proportions of basic amino acids, the molecular ratios of which are so remarkably constant, is probably not without significance; it seems to justify a modification of the definition of a keratin which may be tentatively stated: *A keratin is a protein which is resistant to digestion by pepsin and trypsin, which is insoluble in dilute acids and alkalies, in water and in organic solvents, and which, on acid hydrolysis, yields such quantities of histidine, lysine, and arginine that the molecular ratios of these amino acids are respectively approximately as 1:4:12.*

Keratins usually yield larger proportions of cystine than do other proteins. Data, secured by the colorimetric method (12), are given in Table I which show that the proportion of this amino acid yielded by the different tissues varies between wide limits and bears no definite relationship to the proportions of basic amino acids. A high proportion of cystine is therefore not necessarily characteristic of the proteins usually designated as keratins.

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IPOMŒIN, A GLOBULIN FROM SWEET POTATOES, IPOMŒA BATATAS

ISOLATION OF A SECONDARY PROTEIN DERIVED FROM IPOMŒIN BY ENZYMIC ACTION

By D. BREESE JONES AND CHARLES E. F. GERSDORFF

*(From the Protein and Nutrition Division; Bureau of Chemistry and Soils,
United States Department of Agriculture, Washington)*

(Received for publication, July 3, 1931)

As far as we are aware, there are no data in the literature relative to the proteins of sweet potatoes. Sweet potatoes rank second in importance as a truck crop in the United States and constitute one of the chief vegetable foods in the Southern States. Compared with other root vegetables they rank high in protein, the protein content ranging from 2 to 3.5 per cent. Sweet potatoes contain more true protein than do potatoes, although these two articles are usually represented as containing about the same percentages of crude protein ($N \times 6.25$). About half of the nitrogen in potatoes does not represent protein, but is present largely in the form of amides. No amides have been found in sweet potatoes.

The work described in this paper relates to the isolation and properties of a globulin which constitutes the greater part of the protein content of sweet potatoes. The composition and properties of another protein are described, which is formed from the original globulin by the action of proteolytic enzymes during the aging of the sweet potatoes at temperatures favorable to metabolic activity.

When the juice or sodium chloride extract of recently harvested sweet potatoes, or of those which have been properly stored,¹ is

¹ The sweet potatoes referred to in this paper as having been properly stored were those obtained from the Department of Agriculture Experiment Farm at Arlington, Virginia. After having been harvested they were held for 10 days at 29° (curing period) and then stored at 15–18° at relatively low humidity.

heated there is no separation of a coagulum until the temperature reaches 82°. Continued heating of the filtrate from this coagulum up to boiling yields no further separation. This globulin can also be obtained by dialysis, or by bringing the juice or extract to pH 4 by addition of acetic acid. The precipitation by acidification is not quite complete, however, because the globulin is partly soluble in dilute acid.

When the juice or salt extract of sweet potatoes is allowed to stand for 2 or 3 days at room temperature (20–25°) fermentation takes place, and sufficient acidity develops to cause a spontaneous precipitation of the protein.

When precipitated by acidification, the protein will not redissolve in 5 per cent sodium chloride until enough sodium hydroxide has been added to make the suspension neutral to litmus. The protein thus redissolved is again coagulable at 82° and can be reprecipitated by dialysis, or by addition of ammonium sulfate to 65 per cent of saturation. The general behavior of this protein corresponds to that of a globulin. Because it represents the chief protein of sweet potatoes the name *ipomæin* is suggested.

More than thirty preparations of ipomæin were made from different varieties of sweet potatoes by various combinations of the methods referred to. The elementary composition of all these preparations was determined, and eight of them were analyzed by the Van Slyke method for the distribution of nitrogen.

Material Used

Most of the varieties² of sweet potatoes used in this investigation were grown on the Department of Agriculture Experiment Farm by the Division of Horticultural Crops and Diseases of the Bureau of Plant Industry of the United States Department of Agriculture.³ A few varieties were purchased in the open market.

Some of the sweet potatoes were used soon after harvesting;

² The following varieties were used; Yellow Jersey, Porto Rican Sweets, Nancy Hall, Big Stem Jerseys, Dooley, Triumph, Southern Queen, and Bermuda Red.

³ Acknowledgment is made to J. H. Beattie of the Bureau of Plant Industry for his cooperation in furnishing us with sweet potatoes, both the freshly harvested and those held for different periods under definite storage conditions.

others, used for purposes referred to later, were held in storage for varying periods under definite conditions.

The nitrogen content of five of the seven varieties grown on the Arlington Farm ranged from 0.41 per cent to 0.46 per cent. One variety contained 0.37 per cent and the other had 0.24 per cent. Four varieties obtained in the open market contained 0.25, 0.30, 0.47, and 0.25 per cent nitrogen, respectively. The variations in the nitrogen content of the different samples were probably due to the effect of different soil and climatic conditions. No significant differences were observed in the nitrogen content of samples of the same lot when analyzed soon after digging or after storing for periods ranging from 2 to 6 months.

Preparation and Properties of Ipomæin

5 kilos of finely pulped sweet potatoes (fresh stock) were suspended in 7 to 8 liters of 5 per cent sodium chloride solution. After standing for 1 or 2 hours the mixture was pressed in a fruit press, and the extract was allowed to stand until most of the starch had settled. The liquid was then filtered by suction through a mat of pulped filter paper, and the clear filtrate was acidified to pH 4 by addition of acetic acid. The precipitate which separated was dissolved by suspending it in 5 per cent sodium chloride solution followed by addition of 0.1 N sodium hydroxide solution until the mixture was neutral to litmus. The clear filtered solution was saturated by addition of solid ammonium sulfate. The precipitated protein was separated by filtration and redissolved by addition of distilled water. Enough ammonium sulfate was retained in the precipitate to cause it to dissolve when the water was added. The filtered solution was then dialyzed for 7 days against distilled water. The protein which precipitated was again redissolved by suspension in 5 per cent sodium chloride solution followed by addition of sodium hydroxide to the point of neutrality to litmus. The filtered solution was then heated to 82°. The coagulum was removed by filtration, and was repeatedly washed with boiling water. It was finally dried with alcohol and ether in the usual way. The yield was 5 gm.

The ipomæin thus prepared consisted of a grayish white powder which on incineration gave 0.2 per cent ash. The elementary

composition, percentages of amino acids, and distribution of nitrogen are given in Tables I and II.

Ipomœin separates as a flocculent precipitate from a 5 per cent sodium chloride solution at 65 per cent of saturation with ammonium sulfate, and readily redissolves in sodium chloride solution.

TABLE I

Comparison of Composition of Sweet Potato Proteins with That of Tuberin, the Globulin of the White Potato

The percentages are expressed in terms of ash- and moisture-free proteins.*

	Ipomœin	Secondary protein	Tuberin (white potato)
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Carbon	51.79	53.50	53.61†
Hydrogen	7.19	6.53	6.85†
Nitrogen	16.16	15.26	16.24†
Sulfur	2.25	1.73	1.25†
Amide N*	8.87	8.44	9.24‡
Cystine	2.65, § 1.42	2.49, § 1.62	
Arginine	6.13§	4.79§	4.20‡
Histidine	3.19§	2.50§	2.30‡
Lysine	4.90§	4.98§	3.30‡
Tryptophane	2.69¶	4.78¶	
Tyrosine	7.03**	6.57**	4.30‡

* The values given for amide nitrogen are expressed in percentages of the total nitrogen.

† Osborne and Campbell (1).

‡ Sjollem and Rinkes (2).

§ Determined by the Van Slyke method.

|| Determined colorimetrically by the method of Sullivan (3).

¶ Determined colorimetrically by the method of May and Rose (4).

** Determined colorimetrically by the method of Folin and Ciocalteu (5).

However, when precipitated either by dialysis, or by acidification, it will not redissolve in sodium chloride solution until the suspension is made neutral to litmus by addition of alkali.

Comparison of Ipomœin with Tuberin, the Globulin of Potatoes

For comparison the elementary composition and amino acid percentages of tuberin, the globulin of the white potato, are

included in Table I. The most significant difference in the elementary composition of these two proteins lies in their sulfur content, that of ipomœin being nearly twice that of tuberin. The percentages of amino acids given for the two globulins are not strictly comparable. Those for arginine, histidine, and lysine in ipomœin were determined by the Van Slyke method, whereas the figures given for these amino acids in tuberin were determined by Sjollema and Rinkes (2) by the method of Kossel and Patton. In general

TABLE II

*Distribution of Nitrogen in the Sweet Potato Proteins as Determined by the Van Slyke Method (Expressed in Percentages of Total Nitrogen)**

Nitrogen	Ipomœin†	Secondary protein†
	<i>per cent</i>	<i>per cent</i>
Amide N.....	8.87	8.44
Humins adsorbed by lime.....	2.16	3.07
“ “ in amyl alcohol-ether extract.....	0.47	0.11
Arginine N.....	12.20	10.08
Cystine N.....	1.91	1.91
Histidine N.....	5.36	4.43
Lysine N.....	5.82	6.26
Amino N of filtrate.....	60.44	50.98
Non-amino N of filtrate.....	2.04	13.98
Total N regained.....	99.27	99.26

* Nitrogen figures are corrected for the solubilities of the phosphotungstates of the bases. They represent the average results of closely agreeing duplicate determinations.

† Nitrogen content of ipomœin, 16.16 per cent; nitrogen content of secondary protein, 15.26 per cent.

the results given by the Van Slyke method are higher than those obtained by the method of Kossel and Patton. The percentage found by Sjollema and Rinkes for cystine in tuberin (4.4 per cent) is not included in Table I because this figure is based on the total sulfur content of the protein and is probably too high, for it is unlikely that all the sulfur in potatoes is cystine sulfur. The percentage of tyrosine given for tuberin is doubtless too low. This determination was made by isolation and rarely, if ever, can all the tyrosine be separated in this way. The percentages of trypto-

phane and tyrosine in ipomœin were determined colorimetrically. Two values are given for cystine, one as determined by the Van Slyke method and the other by Sullivan's colorimetric method. The value determined colorimetrically we consider the more accurate.

Formation of a Secondary Protein from Ipomœin as a Result of Enzymic Action

As already referred to, ipomœin was the only protein which we were able to isolate from newly harvested sweet potatoes or from those that had been properly stored. Another protein with properties distinctly different from ipomœin was obtained, however, from sweet potatoes which had remained at ordinary room temperature for several weeks. Evidence was obtained showing that this second protein is an intermediate product formed by the degradation of ipomœin as a result of enzymic action. Increasing quantities of the secondary protein are developed as sweet potatoes are allowed to remain under conditions favorable for enzymic action. At the same time the quantity of ipomœin gradually decreases, as does also the total quantity of coagulable protein. As a result of continued enzymic action the intermediate protein also undergoes degradation, yielding simpler non-coagulable nitrogenous products.

As in seeds, the protein in sweet potatoes is stored as a reserve food for the young growing plant. When the sweet potatoes are held for a period under conditions favorable for the development of cell activity preliminary to sprouting, the protein originally stored is gradually broken down by enzymic action in successive stages of degradation until simple products such as amino acids and amides are formed which can be transported and assimilated by the growing plant. Newton (6) has shown that as a result of proteolytic enzyme activity white potatoes stored at room temperature develop increasing amounts of amino nitrogen as the length of storage increases. Appleman and Miller (7) found that the percentage of non-protein nitrogen in white potatoes increased during storage. An increase in acid amide nitrogen was also noted.

The same secondary protein which was isolated from improperly stored sweet potatoes was also obtained directly from isolated and

purified ipomœin by enzymic decomposition. Although not normally present in fresh sweet potatoes, it can be obtained from them by allowing the juice or saline extract to stand for a few days at room temperature.

Isolation of the Secondary Protein

The juice or sodium chloride extract of sweet potatoes which have been stored at relatively high temperatures, behaves the same as that of fresh sweet potatoes when heated. At 82° ipomœin flocculates, and no further separation of protein occurs on heating the filtrate to boiling. However, if the filtrate from the ipomœin is acidified to pH 4 by addition of acetic acid and then reheated a protein coagulates at 68°. When working with fresh, or properly stored sweet potatoes, on the other hand, no coagulum can thus be obtained from the filtrate from ipomœin.

The secondary protein, when present, can also be obtained from sweet potato extract by removing the ipomœin by acidification, and then heating the filtrate to 68°.

It was also obtained by allowing a sodium chloride solution of carefully purified ipomœin to remain at 20–25°. Increasing quantities of the secondary protein were formed, reaching a maximum in about 2 days. When the acidity which developed in the mixture during this time was reduced to pH 5 by addition of sodium hydroxide, and the mixture was allowed to remain for another 24 hours, a decided increase in the formation of the secondary protein occurred. Only about 20 per cent of the ipomœin originally present in the solution was found to remain. Inasmuch as a purified ipomœin preparation was used in these experiments the question may be raised as to how the changes described could be accounted for on the basis of enzymic action. The ipomœin used had been separated from a 5 per cent sodium chloride extract of sweet potatoes by precipitation with ammonium sulfate, and by subsequent dialysis. It has been previously shown that enzymes present in naturally occurring protein material may be carried along with the proteins during precipitation by ammonium sulfate and dialysis. Jones, Gersdorff, and Moeller (8) showed that the crude protein obtained from the bark of *Robinia pseudacacia* by precipitation either by ammonium sulfate or by alcohol, contained urease and an enzyme capable of hydrolyzing amygdalin. Re-

peated precipitation of the protein by ammonium sulfate and dialysis failed to diminish the enzymic activity associated with the protein.

Eleven preparations of the protein coagulating at 68° agreed closely with respect to their elementary composition. The composition, distribution of nitrogen, and percentages of amino acids in a representative preparation of this protein are given in Tables I and II. Compared with ipomoein, this protein contains less nitrogen and sulfur, but nearly twice as much tryptophane. It cannot be separated from sodium chloride solution by dialysis, but it is precipitable by addition of ammonium sulfate to 90 per cent of saturation. When in acid solution at pH 4 the protein can be precipitated by ammonium sulfate at 80 per cent of saturation. Most of its properties are like those of an albumin.

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STROPHANTHIN

XXIV. ISOMERIC HEXAHYDRODIANHYDROSTROPHANTHIDINS AND THEIR DERIVATIVES

BY WALTER A. JACOBS, ROBERT C. ELDERFIELD, ALEXANDER
HOFFMANN, AND THOMAS B. GRAVE

(*From the Laboratories of The Rockefeller Institute for Medical Research,
New York*)

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The correlation of the digitalis aglucones with periplogenin and strophanthidin has recently been described.¹ This was accomplished by the use of certain derivatives of the iso compounds of these substances. For some time before this group of derivatives was used another method of approach had been attempted, based upon transformations with dianhydrostrophanthidin. Starting with this unsaturated substance the plan had been to hydrogenate its three double bonds with the production of a saturated hexahydrodianhydrostrophanthidin. The next step was the reduction of the carbonyl group still present in the substance to methyl. At this point it was hoped that comparisons with similar substances obtained from other cardiac aglucones might be feasible. A part of our experience with this program has already been presented.² It was found that the catalytic hydrogenation with palladium black of dianhydrostrophanthidin under the conditions which we had used proceeded beyond the stage desired and that an oxygen atom was removed with the formation of a mixture of isomeric octahydrotrianhydrostrophanthidins. This result was correctly interpreted to be due to the simultaneous reduction of the lactol form of the hydroxyaldehyde to the saturated alkylene oxide.

Windaus, Reverey, and Schwieger,³ who were simultaneously

¹ Jacobs, W. A., and Elderfield, R. C., *J. Biol. Chem.*, **91**, 625 (1931); **92**, 313 (1931).

² Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, **63**, 123 (1925).

³ Windaus, A., Reverey, G., and Schwieger, A., *Ber. chem. Ges.*, **58**, 1509 (1925).

occupied with the same problem, later published a report of their own studies. Among other things it was found that dianhydrostrophanthidin, depending upon the catalyst used (platinum black containing hydrogen or oxygen), could be reduced to a hexahydrodianhydrostrophanthidin, $C_{23}H_{34}O_4$, or an octahydro derivative, $C_{23}H_{36}O_4$. In experiments which we have since attempted with various platinum catalysts we have always noted a steady absorption, of course with definite breaks in the curve, of 4 mols of hydrogen with the formation of mixtures of isomeric octahydrotrianhydrostrophanthidins, thus repeating our earlier experience with palladium black. We have not, therefore, been able to duplicate the conditions which were employed by Windaus and coworkers. For this we have no explanation.

In order to prevent the reduction of the lactol to the oxide we turned to the direct hydrogenation of the ethylal of oxidodianhydrostrophanthidin in a neutral solvent in which the ethylal group is stable. In alcoholic solution this substance readily absorbed 3 mols of hydrogen with the formation of a mixture of more soluble isomeric saturated substances. Among these a substance preponderated of melting point 128° and of $[\alpha]_D = +21^\circ$, which has been designated the *ethylal of α -hexahydrooxidodianhydrostrophanthidin*.⁴ On hydrolysis this substance readily yielded the hydroxyaldehyde, *α -hexahydrodianhydrostrophanthidin*. The latter was found to react both in the free aldehydic and in the lactol form. From the fact that in the preparation of its *oxime* more vigorous treatment was necessary than has usually been required by this class of substances the impression was obtained that the lactol form is the more stable form. When the attempt was made to reduce the substance by the Clemmensen method the desired desoxo derivative was not produced. Instead, the substance was reduced in the lactol form with the production of a mixture of isomeric *octahydrotrianhydrostrophanthidins* which resembled in properties the mixture obtained by catalytic hydrogenation. On the other hand, on benzylation the secondary alcoholic group of the aldehydic form of dianhydrostrophanthidin was acylated since the resulting *dianhydrostrophanthidin benzoate* formed an *oxime*.

⁴ The designation of α and β in these cases is arbitrary and bears no relationship to the designation of α and β previously given to the isomeric isostrophanthidins and their derivatives.

With the hope that the benzoyl group would prevent oxide formation the attempt was then made to reduce this benzoate by the Clemmensen method. Although a reaction occurred, the results obtained were inconclusive. The attempt was also made to prepare a semicarbazone for subsequent reduction by the Wolff method. The semicarbazone formation, however, proved to be very incomplete, apparently due to the persistence of the substance in the lactol form under the conditions used. Further investigation of this phase of the problem was, therefore, discontinued especially in view of the greater promise of the work in the isostrophanthidin series, which has since been published.

In the course of the investigation of α -hexahydrodianhydrostrophanthidin, its behavior on oxidation with chromic acid was studied. This reaction was found to proceed smoothly with the formation of a neutral *dilactone*, $C_{23}H_{32}O_4$, from the lactol form. This oxidation product consisted mainly of a substance which differed in melting point and properties from a previously described hexahydrodilactone⁵ which was obtained by hydrogenation of the dianhydrodilactone, $C_{23}H_{26}O_4$. A minor portion of the above oxidation mixture, however, was separated because of its sparing solubility. It appeared to be identical with the earlier saturated dilactone. The formation, therefore, of such isomeric dilactones on oxidation suggested the possibility that the so called α -hexahydrodianhydrostrophanthidin is not entirely homogeneous. This point will, however, be again referred to.

Since the direct hydrogenation of the three double bonds in a substance like the ethylal of oxidodianhydrostrophanthidin is practically certain to give rise to a mixture of isomers another graduated procedure was studied in which each double bond was individually produced and hydrogenated. This was made possible by starting with dihydrostrophanthidin. The latter as already reported⁶ readily loses 1 mol of water with the formation of anhydrodihydrostrophanthidin. This substance was hydrogenated with difficulty to form *dihydroanhydrodihydrostrophanthidin*. The remaining tertiary hydroxyl group was readily removed from this substance by alcoholic hydrogen chloride with the formation of the *ethylal of dihydrooxidodianhydrodihydrostrophanthi-*

⁵ Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, **65**, 504 (1925).

⁶ Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, **74**, 791 (1927).

din. The latter on hydrogenation absorbed 1 mol of hydrogen with the formation of the saturated *ethylal of β -hexahydrooxidodianhydrostrophanthidin*. This substance of melting point 131° and of rotation $[\alpha]_D = +13^{\circ}$ resembles very closely in all other properties the previous α -isomer. On hydrolysis *β -hexahydrodianhydrostrophanthidin* was formed. Like its α -isomer this substance also reacted in both the free aldehydic and the lactol forms. It yielded an *oxime*. Its monobenzoate proved to be likewise a derivative of the aldehydic form since this derivative also gave an *oxime*. Attempts to reduce the benzoate by the Clemmensen method were again inconclusive. Like its α -isomer, *β -hexahydrodianhydrostrophanthidin* was oxidized by chromic acid in the lactol form to give a hexahydrodilactone which again proved to be a mixture. In this case the high melting, sparingly soluble substance predominated which was encountered as a minor fraction of the oxidation product from the α -isomer. This observation brings again in question the homogeneity of the above β -hydrogenation product. It is conceivable though that partial isomerization may have occurred under the conditions of the oxidation. Both of the isomeric hexahydrodianhydrostrophanthidins as well as the dilactones obtained from them were found to be transformed readily into mixtures of isomers on heating with dilute hydrochloric acid.

From our experience with the hydrogenation of the unsaturated strophanthidin derivatives and from that of Windaus and co-workers with the digitalis aglucones,⁷ it is apparent that this method of approach for the correlation of the cardiac aglucones has proved to be too complicated to be practicable. As we have already shown, the iso derivatives of these aglucones have presented much more favorable opportunities. The further use of the iso compounds is being applied in the case of certain other aglucones of the strophanthidin group for purposes of correlation.

EXPERIMENTAL

Part I

Ethylal of α -Hexahydrooxidodianhydrostrophanthidin—A suspension of the ethylal of oxidedianhydrostrophanthidin in alcohol

⁷ Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **86**, 200 (1930).

was shaken with purified hydrogen and 0.1 gm. of platinum oxide catalyst. The absorption, at first rapid, gradually decreased so that in some cases it was necessary after a day or so to add a further 0.1 gm. of catalyst to complete the reaction. In different experiments from 1 to 6 days were found necessary for the absorption of the required 3 mols of hydrogen. The resulting clear solution no longer gave a nitroprusside reaction. The filtrate from the catalyst showed no appreciable acidity so that no evidence was obtained of the formation of desoxy acid during the hydrogenation of the unsaturated lactone group. The filtrate on concentration yielded successive crops of crystals. On fractionation from alcohol about two-thirds of the material was obtained with constant melting point and rotation. It formed needles which were soluble in the usual solvents and melted at 128–129°.

$[\alpha]_D^{20} = +21^\circ$ ($c = 1.0$ in 95 per cent alcohol).

3.880 mg. substance: 3.290 mg. H_2O , 10.580 mg. CO_2 .

$C_{26}H_{35}O_4$. Calculated. C 74.57, H 9.52

Found. " 74.37, " 9.49

The mother liquors yielded additional crystalline material, of somewhat lower melting point and lower rotation, which consisted of stereoisomers.

The above ethylal is isomeric with the substance obtained by the stepwise dehydration and hydrogenation of dihydrostrophanthidin. It appeared possible that this isomerism was due to the interposition of the action of HCl at one of the steps in the series of reactions giving the ethylal from dihydrostrophanthidin. To test this possibility the attempt was made to convert the above ethylal into this latter isomer by refluxing for 30 minutes with 5 per cent absolute alcoholic hydrogen chloride. The solution was then poured into $NaHCO_3$ solution and the precipitate was collected with water. The material proved to be a mixture of substances which crystallized slowly from the concentrated alcoholic solution. This substance proved to be very soluble and melted draggily at 110–115°. Although after recrystallization this melting point could be raised giving a substance of $[\alpha]_D = +14^\circ$, this was accomplished only by a profitless use of material. Although the above ethylal is definitely isomerized by acid the reaction is

not a homogeneous one leading to the isomer derived from dihydro-strophanthidin.

α-Hexahydrodianhydrostrophanthidin—0.5 gm. of the ethylal was dissolved by gentle warming in 4 cc. of 50 per cent acetic acid. After being chilled a precipitate formed on dilution. On recrystallization from methyl alcohol the hydroxyaldehyde formed fine needles which softened at about 112° to a resinous mass of bubbles that finally melted at 178–180°.

$$[\alpha]_D^{25} = +41^{\circ} \text{ (c = 1.055 in chloroform).}$$

For analysis the substance was dried at 80° and 15 mm.

5.147 mg. substance: 4.295 mg. H₂O, 13.960 mg. CO₂.
 C₂₃H₃₄O₄. Calculated. C 73.74, H 9.16
 Found. " 73.97, " 9.34

This hydroxyaldehyde is apparently a mixture of the lactol and free aldehydic forms. The formation of an *oxime* did not proceed with the usual readiness. For a complete reaction it was found necessary to heat the hydroxyaldehyde in alcoholic solution with hydroxylamine hydrochloride and sodium acetate in a sealed tube at 100° for 6 hours. The reaction product obtained on dilution was recrystallized from alcohol and formed leaflets which melted at 258–260° after preliminary softening.

4.498 mg. substance: 3.585 mg. H₂O, 11.723 mg. CO₂.
 6.520 " " : 0.211 cc. N (28°, 754.5 mm.).
 C₂₃H₃₃O₄N. Calculated. C 70.90, H 9.07, N 3.59
 Found. " 71.07, " 8.92
 " " 3.65

Octahydrotrianhydrostrophanthidin—When the attempt was made to apply the Clemmensen method for the reduction of *α*-hexahydrodianhydrostrophanthidin to the desoxo compound the reaction took a different course. The substance was reduced directly in the lactol form to the saturated oxide. For this purpose the ethylal was directly used because of its prompt preliminary hydrolysis.

A solution of 0.1 gm. of the ethylal in 5 cc. of acetic acid was treated with 2.5 gm. of amalgamated zinc and 2.5 cc. of HCl (1.19). The mixture was refluxed for ½ hour. The reaction product crys-

tallized on dilution. On fractionation from methyl alcohol it was found to be a mixture of isomers. The first crop which was still apparently a mixture melted at 196–202° and gave a rotation of $[\alpha]_D^{26} = +61^\circ$ ($c = 0.990$ in chloroform). This material closely resembles the substance previously obtained by catalytic hydrogenation of dianhydrostrophanthidin.² No attempt was made to purify it exhaustively.

5.120 mg. substance: 4.470 mg. H₂O, 14.540 mg. CO₂.
 C₂₀H₁₄O₃. Calculated. C 77.03, H 9.57
 Found. " 77.45, " 9.77

The oxidic character of the substance was shown by the fact that it was not attacked under the usual conditions by permanganate or chromic acid. The above mother liquors yielded lower melting isomeric material with similar properties.

Benzoyl- α -Hexahydrodianhydrostrophanthidin—The benzoate was prepared by benzylation in pyridine solution. It formed very sparingly soluble crystalline aggregates from alcohol which melted at 213–215°.

4.520 mg. substance: 3.250 mg. H₂O, 12.475 mg. CO₂.
 C₃₀H₁₈O₅. Calculated. C 75.27, H 8.01
 Found. " 75.27, " 8.05

This substance was shown to be the benzoyl derivative of the hydroxyaldehyde and not its lactol form by its ready conversion into an *oxime*. This derivative was made in the usual manner in alcoholic solution. It formed needles from alcohol which melted at 242–243°.

4.513 mg. substance: 3.252 mg. H₂O, 12.043 mg. CO₂.
 6.548 " " : 9.174 cc. N (24°, 760 mm.).
 C₃₀H₁₈O₅N. Calculated. C 72.97, H 7.97, N 2.84
 Found. " 72.77, " 8.06
 " " 3.13

In all attempts to prepare a semicarbazone from the benzoate, difficulty was experienced in bringing the reaction to completion. The substance recovered was never homogeneous and no satisfactory means of purification were found.

Hexahydrodilactone, C₂₂H₃₂O₄—Since hydrolysis of the ethylal group readily occurred, the ethylal was directly employed for

oxidation to the dilactone. 0.5 gm. of the α -hexahydroethylal was dissolved in 10 cc. of acetic acid and the solution was treated with 2.4 cc. of Kiliani CrO_3 solution. A prompt reaction occurred. After 10 minutes the mixture was diluted. Fractionation from alcohol was necessary in order to remove small amounts of higher melting, less soluble, isomeric material. The majority was recovered as narrow prisms or needles from alcohol, which melted at $192\text{--}194^\circ$. The homogeneity of this fraction is perhaps doubtful.

$[\alpha]_D^{25} = +19.4^\circ$ ($c = 0.515$ in chloroform).

4.305 mg. substance: 3.440 mg. H_2O , 11.685 mg. CO_2 .

$\text{C}_{23}\text{H}_{32}\text{O}_4$. Calculated. C 74.14, H 8.67

Found. " 74.02, " 8.94

The small fraction of less soluble isomer obtained during the above fractionation was recrystallized from acetone. It formed needles and short stout prisms which melted at $265\text{--}267^\circ$.

$[\alpha]_D^{25} = +13^\circ$ ($c = 1.10$ in chloroform).

4.562 mg. substance: 3.580 mg. H_2O , 12.420 mg. CO_2 .

Found. C 74.25, H 8.78

This substance is apparently identical with the previously reported hexahydrodilactone⁵ obtained by hydrogenation of the dianhydrodilactone, $\text{C}_{23}\text{H}_{26}\text{O}_4$. Since H_2SO_4 was present in the Kiliani CrO_3 solution used above, it appeared possible that the small amount of high melting isomer might have been due to the isomerizing effect of the strong acid. This appears to have been confirmed by the following experiment.

0.2 gm. of the hexahydrodilactone melting at $192\text{--}194^\circ$ was refluxed in a mixture of 25 cc. of acetic acid and 5 cc. of HCl (1.19) for 45 minutes. Crystalline material was obtained on dilution. After several recrystallizations from methyl alcohol and finally from acetone it melted at $261\text{--}264^\circ$.

$[\alpha]_D^{25} = +13.2^\circ$ ($c = 0.740$ in chloroform).

4.597 mg. substance: 3.565 mg. H_2O , 12.510 mg. CO_2 .

Found. C 74.17, H 8.67

On this occasion we have checked more fully the previously reported⁵ direct hydrogenation of the dianhydrodilactone, $\text{C}_{23}\text{H}_{26}\text{O}_4$. The reaction product has been found to be also a mixture of

isomers. As previously given repeated recrystallization from alcohol gave the substance melting at 265–267° with $[\alpha]_D = +14^\circ$. The mother liquors from this material yielded more soluble fractions from which an isomer has now been separated by taking advantage of its greater solubility in ether. It was finally obtained by concentration of the ether solution as characteristic six-sided platelets which melted at 204°. This melting point depended upon the solvent since when alcohol was used it melted at 196–199°.

$[\alpha]_D^{25} = +18^\circ$ ($c = 1.03$ in chloroform).

5.320 mg. substance: 4.185 mg. H_2O , 14.495 mg. CO_2 .

Found. C 73.95, H 8.80

Although this hexahydrodilactone resembles so closely in properties the above low melting oxidation product of the hexahydroethylal it must be a different substance. Contrary to the latter, it was not converted into a high melting isomer on boiling in acetic acid containing HCl.

Part II

Benzoyl Monoanhydrodihydrostrophanthidin—This substance was prepared by benzoylation of monoanhydrodihydrostrophanthidin⁶ in pyridine solution. It formed stout prisms which melted, at 190–192°.

0.0963 gm. substance: 0.0644 gm. H_2O , 0.2570 gm. CO_2 .

$C_{19}H_{18}O_4$. Calculated. C 73.13, H 7.37

Found. " 72.87, " 7.48

Dihydromonoanhydrodihydrostrophanthidin—The double bond of monoanhydrodihydrostrophanthidin was found to be unusually difficult to hydrogenate. Only when very carefully performed was the operation a smooth and complete affair. The best results were obtained by the use of material which had been repeatedly recrystallized from alcohol that had been distilled repeatedly in a glass jointed apparatus.

A suspension of 6.2 gm. of substance in 400 cc. of alcohol was shaken with hydrogen and 0.2 gm. of the platinum catalyst of Adams and Shriner. In this instance absorption corresponding to 1 mol required several hours for completion. On another

occasion $1\frac{1}{2}$ hours sufficed while in other attempts it was necessary to add more catalyst and even then several days were required before the absorption of 1 mol was completed.

The saturated substance formed leaflets from methyl alcohol which melted at $217-219^{\circ}$.

$[\alpha]_D^{20} = +28^{\circ}$ ($c = 1.000$ in pyridine).

0.1005 gm. substance: 0.0789 gm. H_2O , 0.2613 gm. CO_2 .

$C_{23}H_{34}O_5$. Calculated. C 70.72, H 8.78

Found. " 70.89, " 8.79

Ethylal of Dihydrooxidodianhydrodirostrophanthidin—The preceding hydroxyaldehyde was refluxed for 30 minutes in 10 parts of absolute alcohol containing 5 per cent of hydrogen chloride. On being cooled the mixture formed a thick mass of crystals. The ethylal formed stout needles from alcohol and melted at $174-176^{\circ}$.

$[\alpha]_D^{25} = -108^{\circ}$ ($c = 0.950$ in chloroform).

2.946 mg. substance: 2.346 mg. H_2O , 8.075 mg. CO_2 .

$C_{23}H_{36}O_4$. Calculated. C 74.94, H 9.06

Found. " 74.74, " 8.91

Dihydroodianhydrodihydrostrophanthidin—On diluting an alcoholic solution of the above ethylal with water containing dilute hydrochloric acid hydrolysis occurred and the free hydroxyaldehyde slowly separated as arborescent masses of prisms which were characterized by great crystallizing power. It formed sparingly soluble prisms from 95 per cent alcohol which did not exhibit a definite melting point. It slowly softened above 215° but the mass did not become clear until 247° .

4.011 mg. substance: 3.119 mg. H_2O , 10.868 mg. CO_2 .

$C_{21}H_{32}O_4$. Calculated. C 74.14, H 8.86

Found. " 73.89, " 8.70

Ethylal of β -Hexahydrooxidodianhydrostrophanthidin—The preceding unsaturated ethylal in ethyl alcoholic solution rapidly absorbed 1 mol of hydrogen when either palladium or platinum catalysts were used. The saturated substance readily crystallized from alcohol as needles which melted at $131-132^{\circ}$.

$[\alpha]_D^{25} = +13.4^\circ$ ($c = 1.466$ in alcohol).

3.917 mg. substance: 3.291 mg. H_2O , 10.681 mg. CO_2 .

$C_{21}H_{28}O_4$. Calculated. C 74.57, H 9.52

Found. " 74.36, " 9.40

This substance resembles the α -isomer in general properties. On heating with alcoholic hydrogen chloride it is also isomerized to a mixture of substances which were not investigated.

β -Hexahydrodianhydrostrophanthidin—The above ethylal was warmed in 10 parts of 50 per cent acetic acid until solution was complete. On dilution and chilling the free hydroxyaldehyde separated. When recrystallized from methyl alcohol it formed needles which melted at 155 – 156° after preliminary softening.

$[\alpha]_D^{25} = +20^\circ$ ($c = 0.705$ in chloroform).

3.687 mg. substance: 2.972 mg. H_2O , 9.955 mg. CO_2 .

$C_{21}H_{24}O_4$. Calculated. C 73.74, H 9.15

Found. " 73.63, " 9.02

This substance reacts in either the free aldehydic or lactol form.

The *oxime* was obtained in the usual manner and crystallized from alcohol as needles which melted at 262° after preliminary softening.

4.270 mg. substance: 3.410 mg. H_2O , 11.102 mg. CO_2 .

6.350 " " : 0.217 cc. N (23° , 755 mm.).

$C_{21}H_{22}O_4N$. Calculated. C 70.90, H 9.06, N 3.60

Found. " 70.90, " 8.93, " 3.90

Like the α -isomer, β -hexahydrodianhydrostrophanthidin is oxidized in the lactol form by Kiliani solution to a *hexahydrodilactone*. This appeared to be also a mixture of isomers in which a sparingly soluble high melting form seemed to predominate. After repeated recrystallization from acetone it formed fine needles which melted at 268 – 269° . It appeared to be identical with the previously described high melting dilactone obtained as a minor fraction of the oxidation product of α -hexahydrodianhydrostrophanthidin.

$[\alpha]_D^{25} = +13.6^\circ$ ($c = 0.825$ in chloroform).

3.943 mg. substance: 3.060 mg. H_2O , 10.770 mg. CO_2 .

$C_{21}H_{22}O_4$. Calculated. C 74.14, H 8.67

Found. " 74.50, " 8.68

Lower melting material which was recovered from the mother liquors was not further investigated.

Benzoyl-β-Hexahydrodianhydrostrophanthidin—Acylation was accomplished with benzoyl chloride in pyridine solution. The product formed sparingly soluble lustrous leaflets from 95 per cent alcohol, which melted at 209–211°.

2.594 mg. substance: 1.842 mg. H₂O, 7.137 mg. CO₂.

C₃₀H₂₈O₆. Calculated. C 75.27, H 8.01

Found. " 75.03, " 7.95

This substance is a derivative of the aldehydic form since it yields an *oxime*. For a complete reaction it was necessary to reflux an alcoholic solution of the reactants for about 5 hours. The oxime formed short prisms from alcohol which melted at 252°.

4.685 mg. substance: 3.255 mg. H₂O, 12.550 mg. CO₂.

5.150 " " : 0.137 cc. N (24°, 750.5 mm.).

C₃₀H₂₉O₆N. Calculated. C 72.97, H 7.97, N 2.84

Found. " 73.05, " 7.76

"

" 3.02

THE HYDROGENATION OF UNSATURATED LACTONES TO DESOXY ACIDS. II

BY WALTER A. JACOBS AND ALBERT B. SCOTT

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York)

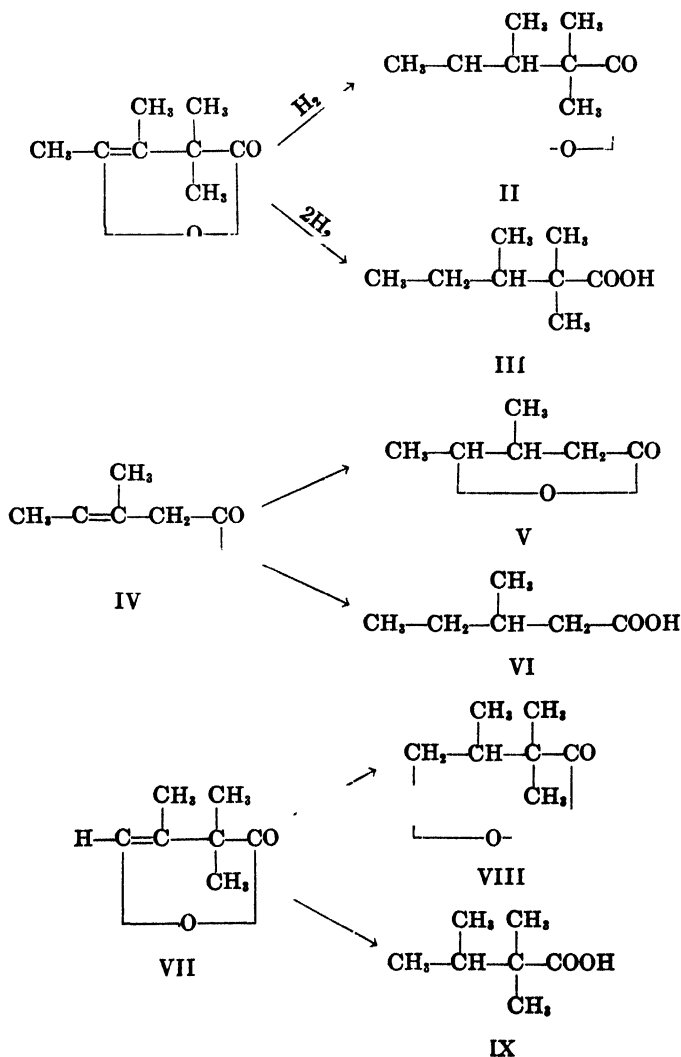
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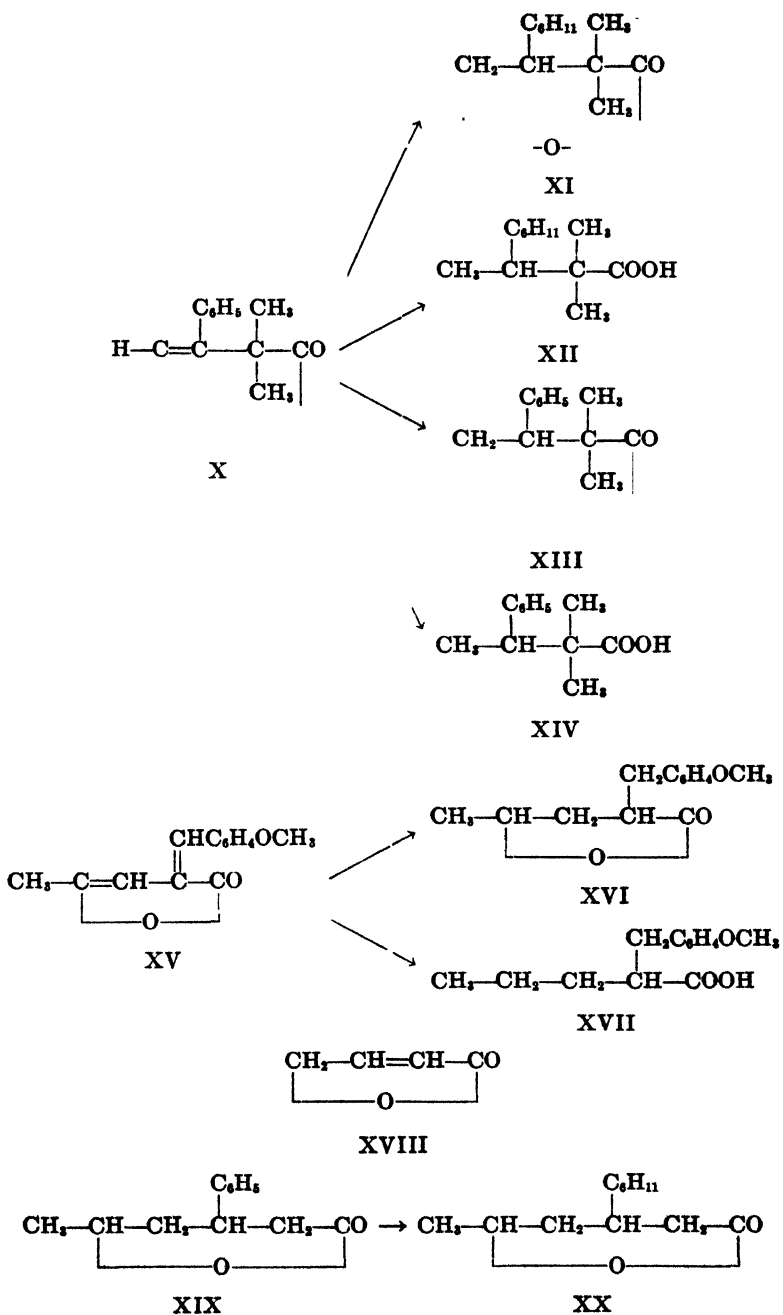
In a previous communication¹ it has been shown that in the case of the unsaturated lactones derived from the enolic forms of certain γ - and δ -keto acids in which the double bond is at the point of lactonization, hydrogenation proceeds with the absorption of 2 mols of H_2 to the saturated desoxy acid. A question which required further investigation, however, was the apparent inconsistency shown in the fact that the strophanthidin group of cardiac aglucones which are $\Delta^{\beta,\gamma}$ -unsaturated γ -lactones appeared on hydrogenation to yield only neutral dihydro derivatives. In the case of strophanthidin² itself under very careful conditions and by the use of a very active catalyst (Adams and Shriner) the difficult additional step of reduction of the aldehyde group to the primary alcohol could be accomplished but without the formation of an appreciable amount of acid. In order to explain the failure of these aglucones to form desoxy acids it became advisable to extend somewhat the study of simpler unsaturated lactones in order to include certain features associated with the unsaturated lactone group of these aglucones. These aglucones are γ -lactones of the enolic form of aldehydo acids. Further, they are substituted in the β -position. We have, therefore, chosen for further study under exactly comparable conditions a few accessible substances which would indicate the effect on previously studied substances first of β -substitution and then of changing from the lactone of a keto acid to that of an aldehydo acid.

¹ Jacobs, W. A., and Scott, A. B., *J. Biol. Chem.*, **87**, 601 (1930).

² Jacobs, W. A., *J. Biol. Chem.*, **88**, 528 (1930).

Whereas α,α -dimethyl- $\Delta^{\beta,\gamma}$ -angelicalactone¹ was previously shown to go completely to the desoxy acid, α,α,β -trimethyl- $\Delta^{\beta,\gamma}$ -angelicalactone (I) yielded roughly only 50 per cent of α,α,β -trimethylvaleric acid (III). The other reaction product was α,α,β -trimethyl- γ -valerolactone (II). A similar result was obtained on hydrogenating β -methyl- $\Delta^{\beta,\gamma}$ -angelicalactone (IV). $\Delta^{\beta,\gamma}$ -Angeli-





calactone³ goes completely to valeric acid. In the case of its β -methyl derivative 70 per cent, at the most, was converted into β -methylvaleric acid (VI). A neutral fraction of β -methyl- γ -valerolactone (V) was also obtained. The experience with these two substances thus indicates that β -substitution in $\Delta^{\beta,\gamma}$ -lactones is a factor which diminishes hydrogenation to desoxy acids.

Opportunity to test the influence of a change from a γ -keto acid lactone to a γ -aldehydo acid lactone was presented by the hydrogenation of α, α, β -trimethyl- $\Delta^{\beta,\gamma}$ -butenolide (VII). In this case the major reaction product was α, α, β -trimethylbutyrolactone (VIII), while only 21 per cent was converted to α, α, β -trimethylbutyric acid (IX). The acid formation here was, therefore, less than half of that observed in the case of the analogous ketolactone.

It might be suggested that the influence of the β -methyl group is only an apparent one and that the observed effect is really due to a partial shift of the double bond to a position between the β -carbon atom and the carbon atom of this methyl group, thus changing the latter to methylene. In such a case a saturated lactone would result perhaps without desoxy acid formation. This explanation, however, is very improbable, especially in view of our experience with another substance where such a shift has been excluded. This substance was α, α -dimethyl- β -phenyl- $\Delta^{\beta,\gamma}$ -butenolide (X). On exhaustive hydrogenation with complete reduction of the aromatic nucleus only about 25 per cent was reduced to α, α -dimethyl- β -cyclohexylbutyric acid (XII). The major reaction product was α, α -dimethyl- β -cyclohexylbutyrolactone (XI). It is practically excluded that the relatively low acid formation in this case was caused by the shifting of double bonds during the hydrogenation of the aromatic nucleus itself. This was shown by the fact that when the reaction was stopped at approximately the 2 mol stage the same proportion of desoxy acid

³ Since our first communication Professor Asahina has kindly brought to our attention his earlier paper (Asahina, Y., and Fujita, A., *Acta Phytchimica*, 1, 1 (1922)) containing observations which appear to have remained generally unknown. In their studies on the structure of anemonin it was noted that α -angelicalactone yields *n*-valeric acid on catalytic hydrogenation. This observation was made to explain the similar catalytic reduction of dihydroanemonin to sebacic acid. These workers appear to be the first to have noted the catalytic reduction of unsaturated lactones to desoxy acids.

and neutral lactone resulted. These substances were respectively α , α -dimethyl- β -phenylbutyric acid (XIV) and α , α -dimethyl- β -phenylbutyrolactone (XIII).

From these results the behavior of the cardiac aglucones as lactones of β -substituted γ -aldehyde acids no longer represents an inconsistency. The failure in these cases of any appreciable acid formation is apparently due to the added effect of the complexity of the portion of the molecule which is here the β -substituent. The β -phenyl substituted lactones which Mannich and Butz⁴ and the writers have found to be completely hydrogenated to desoxy acids are δ -lactones. The latter as a group are more readily opened than γ -lactones. A greater tendency toward reductive cleavage in the case of δ -lactones should tend to counteract any inhibiting effect of β -substitution. The complete reduction of anhydro- γ -isogitoxigenic methyl ester to the desoxy acid⁵ in spite of the fact that its unsaturated lactone group is β -substituted may be explained by the fact that it is a δ -lactone. A similar instance has been recently⁶ noted in the case of the unsaturated δ -lactone anhydride, $C_{21}H_{24}O_6$, obtained from duodephanthondi-acid.

In the course of this work the opportunity was presented to include a few additional unsaturated lactones in the study. In the case of one of these, α -anisyl- Δ^{β} , γ -angelicalactone (XV), the hydrogenation was arbitrarily interrupted after the absorption of 2.4 mols of H_2 (where a definite change in the absorption rate had occurred) to avoid the complication of reduction of the aromatic nucleus. Contrary to α , α -dimethylangelicalactone,¹ this substance gave mostly α -anisyl- γ -valerolactone (XVI) and only 17 per cent of the desoxy acid α -anisylvaleric acid (XVII). In this case the presence of conjugated double bonds unquestionably influenced the course of the reaction. The predominating reaction can be pictured as 1-4 addition to the two double bonds with the intermediate formation of α -anisyl- $\Delta^{\alpha,\beta}$ -angelicalactone which was then reduced to the saturated lactone. With the hope of studying the hydrogenation of the simplest γ -aldehyde acid lactone, Δ^{β} , γ -butenolide (XVIII) was employed since the Δ^{β} , γ -isomer was not

⁴ Mannich, C., and Butz, A., *Ber. chem. Ges.*, **62**, 461 (1929).

⁵ Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **86**, 203 (1930).

⁶ Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **92**, 338 (1931).

available. On hydrogenation this lactone gave roughly 90 per cent of butyrolactone and 10 per cent of butyric acid. A small amount of $\Delta^{\beta,\gamma}$ -butenolide, already present or possibly formed during the hydrogenation, was the probable source of this butyric acid.

In our previous paper it was shown that the lactone of δ -hydroxy- β -phenyl- $\Delta^{\gamma,\delta}$ -hexenoic acid goes completely to the desoxy acid without the apparent formation of an intermediate saturated lactone, β -phenyl- δ -caprolactone (XIX). This was at variance with the results reported by Mannich and Butz⁴ in the case of the lactone of δ -hydroxy- β , δ -diphenyl- $\Delta^{\gamma,\delta}$ -pentenoic acid and the analogous β -methylenedioxyphenyl derivative. Our conclusion has been more recently substantiated by an examination of the behavior of β -phenyl- δ -caprolactone itself. On exhaustive hydrogenation, which involved the aromatic nucleus, this substance yielded but a negligible amount of acid. Practically all was a neutral mixture of stereoisomeric forms of β -cyclohexyl- δ -caprolactone (XX).

EXPERIMENTAL

In the following hydrogenation experiments a constant procedure was used. The solvent was ethyl alcohol which had been distilled three times through an all glass apparatus. The catalyst was the platinum oxide catalyst of Adams and Shriner⁷ prepared, however, with the modification of longer heating recommended by Sigmund.⁸ In all experiments the same lot of catalyst was used. Hydrogenation was carried out at 20–25° and at atmospheric pressure. Gas volumes given are all for the dry gas at normal temperature and pressure and are corrected for the calculated absorption due to the reduction of the oxide catalyst.

Hydrogenation of α, α, β -Trimethyl- $\Delta^{\beta,\gamma}$ -Angelicalactone—Bardhan's method⁹ for the preparation of this lactone, which consisted in the thermal decomposition of β, γ -dibromo- α, α, β -trimethyl-valeric acid, was followed. After several fractionations material was collected of b.p.₁₃ 80–85°, which still gave a positive halogen

⁷ Adams, R., and Shriner, R. L., *J. Am. Chem. Soc.*, **45**, 2171 (1923).

⁸ Sigmund, F., *Sitzungsber. Akad. Wissensch. Wien. Math.-natur. Klasse, 2. Abt.*, **138**, 611 (1929).

⁹ Bardhan, J. C., *J. Chem. Soc.*, 2616 (1928).

test. For purification this material was boiled for 1 hour with 4 parts of quinoline. The mixture was distilled at about 20 mm. until the distillate became red. After the latter was treated with dilute H_2SO_4 in excess, the mixture was extracted with ether. The ether solution after being washed with sodium carbonate solution yielded the halogen-free lactone of b.p.₁₃ 79–80° (Bardhan gives b.p.₁₃ 80°).

3.84 gm. of the lactone were hydrogenated with an initial 60 mg. of catalyst. Since the absorption for some undetermined reason was slow, occasional additional amounts of catalyst were added, making a total of 0.48 gm. After 76 hours, absorption of hydrogen stopped at a total of 965 cc. Calculated for 1 mol of H_2 , 615 cc. This indicated that 57 per cent was reduced to desoxy acid.

Direct titration for free acid against phenolphthalein required 13.2 cc. of *N* NaOH. An excess of alkali was then added to the solution which after being refluxed for 3 hours was titrated back. An additional 16.0 cc. of *N* NaOH were required, or a total of 29.2 cc. Calculated, 27.7 cc. From the direct titration 48 per cent of the lactone was converted into desoxy acid.

The above alkaline solution was concentrated and after being acidified with an excess of H_2SO_4 it was boiled to relactonize hydroxy acid. An ether extract was carefully shaken out with dilute sodium carbonate solution in order to remove trimethylvaleric acid. After the ether solution was washed with saturated NaCl solution it was dried and concentrated. The neutral residue which weighed 1.77 gm. was trimethyl- γ -valerolactone and showed a b.p.₃₃ 121–123°.

4.967 mg. substance: 4.352 mg. H_2O , 12.275 mg. CO_2 .

$\text{C}_8\text{H}_{14}\text{O}_2$. Calculated. C 67.56, H 9.92

Found. " 67.40, " 9.80

The above Na_2CO_3 solution of trimethylvaleric acid was reacidified and extracted with ether. The ether residue on fractionation boiled at 137–138° at 43 mm.

5.940 mg. substance: 5.885 mg. H_2O , 14.490 mg. CO_2 .

$\text{C}_8\text{H}_{16}\text{O}_2$. Calculated. C 66.62, H 11.17

Found. " 66.57, " 11.09

In a second experiment complete absorption of hydrogen was accomplished within 3 hours. In this case the excessive hydrogen absorption over 1 mol was 59 per cent. The amount of free acid found by titration was 52 per cent.

Hydrogenation of β -Methyl- $\Delta^{\beta,\gamma}$ -Angelicalactone—This lactone was prepared from β -methyllevulinic acid by the method used by Wolff¹⁰ in the case of levulinic acid. The keto acid was slowly distilled during 3 hours at ordinary pressure and the distillate was taken up in ether. The dry ethereal solution was concentrated and carefully fractionated. In this manner β -methyl- $\Delta^{\beta,\gamma}$ -angelicalactone, b.p.₁₃ 83–86°, was prepared (Pauly, Gilmour, and Will¹¹ give b.p.₁₃ 87–89°). An alcoholic solution of 1.02 gm. of this lactone with 0.05 gm. of catalyst absorbed 309 cc. of H₂ in 33 minutes. Calculated for 1 mol of H₂, 204 cc. This indicated that 51 per cent of desoxy acid was formed. Direct titration of free acid required 64.0 cc. of N NaOH, or 70 per cent of free acid. A second portion of 1.07 gm. with 0.06 gm. of catalyst absorbed 323 cc. of H₂ in 36 minutes. Calculated for 1 mol of H₂, 214 cc. Direct titration of free acid required 67 cc. of N NaOH. This indicated again 51 and 70 per cent of desoxy acid by the two measurements. For this wide divergence of the hydrogen absorption and the direct titration we have no explanation.

The two portions were combined at this point and required 5.7 cc. of N NaOH for saponification. Total observed alkali equivalent, 18.8 cc. of N NaOH. Calculated, 18.7 cc. The neutral and acidic products were isolated as in the previous case.

The crude neutral fraction which consisted of β -methylvalerolactone was about 0.35 gm. and distilled roughly at 60–70° and 14 mm.

5.144 mg. substance: 4.110 mg. H₂O, 11.935 mg. CO₂.

C₈H₁₀O₂. Calculated. C 63.10, H 8.82

Found. " 63.28, " 8.94

The acid fraction gave 1.5 gm. of crude β -methylvaleric acid, which on distillation showed a b.p.₇₅₈ 197–198° (corrected). The silver salt was prepared for further characterization.

¹⁰ Wolff, L., *Ann. Chem.*, **229**, 251 (1885).

¹¹ Pauly, H., Gilmour, R., and Will, G., *Ann. Chem.*, **403**, 152 (1914).

6.631 mg. substance: 2.930 mg. H_2O , 7.840 mg. CO_2 , 3.220 mg. Ag.
 $C_6H_{11}O_2Ag$. Calculated. C 32.24, H 4.98, Ag 48.39
Found. " 32.25, " 4.94, " 48.56

Hydrogenation of α, α, β -Trimethyl- Δ^2, γ -Butenolide—The lactone was prepared by the procedure of Courtot¹² and Blaise and Courtot,¹³ except that the final removal of halogen was accomplished by boiling with quinoline. This treatment gave halogen-free lactone of b.p.₁₂ 60–63° (Blaise and Courtot give b.p.₁₂ 65°).

1.43 gm. of this lactone were hydrogenated with 0.1 gm. of catalyst. At the end of 73 minutes absorption stopped at 305 cc. of hydrogen. Calculated for 1 mol of H_2 , 254 cc. The excess indicated formation of 20 per cent of desoxy acid. Direct titration of free acid required 2.4 cc. of N NaOH, or 21 per cent of acid formation. For saponification there was required an additional 8.1 cc. of N NaOH, or a total of 10.5 cc. Calculated, 11.3 cc.

After relactonization of the above alkaline solution the neutral and acid fractions were separated as usual. The neutral fraction of α, α, β -trimethylbutyrolactone (0.9 gm.) was distilled at atmospheric pressure and crystallized at room temperature. The b.p. of 204–206° was obtained. (Noyes¹⁴ gives b.p. 211–212°). On redistillation it showed a b.p.₁₄ 92–94°.

7.174 mg. substance: 6.132 mg. H_2O , 17.217 mg. CO_2 .
 $C_7H_{14}O_2$. Calculated. C 65.58, H 9.45
Found. " 65.45, " 9.56

The crude acid fraction (0.3 gm.) of α, α, β -trimethylbutyric acid of b.p.₁₃ 104–105° gave a colorless oil which crystallized readily in the receiver (Haller and Bauer¹⁵ give b.p.₁₃ 104–105°).

5.461 mg. substance: 5.230 mg. H_2O , 12.995 mg. CO_2 .
 $C_7H_{14}O_2$. Calculated. C 64.57, H 10.84
Found. " 64.90, " 10.71

¹² Courtot, A., *Bull. Soc. Chim.*, series 3, **35**, 298 (1906).

¹³ Blaise, E. E., and Courtot, A., *Bull. Soc. Chim.*, series 3, **35**, 995 (1906).

¹⁴ Noyes, W. A., *Am. Chem. J.*, **33**, 362 (1905).

¹⁵ Haller, A., and Bauer, E., *Compt. rend. Acad.*, **149**, 6 (1909).

The silver salt formed slender needles from water.

5.034 mg. substance: 2.458 mg. H_2O , 6.425 mg. CO_2 , 2.287 mg. Ag.

$C_7H_{13}O_2Ag$. Calculated. C 35.43, H 5.45, Ag 45.55

Found. " 34.93, " 5.45, " 45.27

Hydrogenation of α , α -Dimethyl- β -Phenyl- Δ^{β} , γ -Butenolide—This lactone was prepared by the method of Blaise and Courtot.¹⁶ An alcoholic solution of 0.56 gm. of this lactone was hydrogenated with 50 mg. of catalyst. The experiment was interrupted at the end of 202 minutes after 134 cc. had been absorbed. Calculated for 2 mols of H_2 , 134 cc. Direct titration of free acid required 0.73 cc. of N NaOH, or 24 per cent, while after saponification an additional 2.1 cc. of N NaOH were consumed, or a total of 2.83 cc. Calculated, 2.98 cc.

The neutral fraction recovered after relactonization was obtained from dry ether as leaflets which melted at 91–92°. The substance was moderately soluble in ethyl alcohol and petroleic ether and readily soluble in ether, acetone, and benzene. The analytical figures agreed with α , α -dimethyl- β -phenylbutyrolactone.

3.811 mg. substance: 2.590 mg. H_2O , 10.605 mg. CO_2 .

$C_{13}H_{14}O_2$. Calculated. C 75.75, H 7.42

Found. " 75.88, " 7.60

The acid fraction was obtained as an oil which readily crystallized. After recrystallization from dilute acetone the substance melted at 61–62° and was very soluble in ether, acetone, benzene, and petroleic ether. The analytical figures agreed with α , α -dimethyl- β -phenylbutyric acid.

3.510 mg. substance: 2.640 mg. H_2O , 9.668 mg. CO_2 .

$C_{13}H_{16}O_2$. Calculated. C 74.96, H 8.38

Found. " 75.12, " 8.41

In a second experiment the hydrogenation was allowed to go to completion involving the aromatic nucleus. This resulted in the formation of cyclohexyl compounds corresponding to the products of the first experiment. 1.22 gm. of the unsaturated lactone and

¹⁶ Blaise, E. E., and Courtot, A., *Bull. Soc. Chim.*, series 3, **35**, 1002 (1906).

0.14 gm. of catalyst were used. The absorption of H_2 was 639 cc. at the end of 23 hours. Calculated for 4 mols of H_2 , 580 cc. This excess indicated 41 per cent of acid formation. This was considerably higher than that shown by the direct titration for free acid, *i.e.* 1.62 cc. of N NaOH, or 25 per cent. Saponification required an additional 4.0 cc. of N NaOH, or a total of 5.62 cc. Calculated, 6.49 cc.

After relactonization 1.06 gm. of α , α -dimethyl- β -cyclohexylbutyrolactone were obtained. It formed large plates from ether, which melted at 51–52°.

4.090 mg. substance: 3.730 mg. H_2O , 11.00 mg. CO_2 .

$C_{12}H_{20}O_2$. Calculated. C 73.39, H 10.30
Found. " 73.35, " 10.21

The acid fraction of α , α -dimethyl- β -cyclohexylbutyric acid crystallized from dilute acetone as irregular plates which melted at 39–40°.

3.670 mg. substance: 3.682 mg. H_2O , 9.753 mg. CO_2 .

$C_{12}H_{20}O_2$. Calculated. C 72.65, H 11.20
Found. " 72.48, " 11.23

Hydrogenation of α -Anisal- Δ^{β} , γ -Angelicalactone—The lactone was prepared by the method of Thiele.¹⁷ For 4.4 gm. of this lactone suspended in alcoholic solution 0.15 gm. of catalyst was used. The hydrogenation was interrupted after 3 hours when there was a definite slowing up of the rate of absorption. This was done to avoid the possible complication of hydrogenation of the aromatic nucleus. The observed hydrogen absorption was 1087 cc. Calculated for 2 mols of H_2 , 912 cc. Direct titration of free acid required 3.5 cc. of N NaOH, while saponification with an excess of alkali required an additional 19.7 cc. of N NaOH, or a total of 23.2 cc. Calculated, 20.4 cc.

2.6 gm. of neutral material were recovered after relactonization and proved to be α -anisyl- γ -valerolactone. On recrystallization from petroleic ether it formed glistening plates which melted at 60–61° and were soluble in the usual solvents. The saturated

¹⁷ Thiele, J., Tischbein, R., and Lossow, E., *Ann. Chem.*, **319**, 185 (1901).

character of the substance was shown by its behavior toward bromine and permanganate.

4.098 mg. substance: 2.595 mg. H_2O , 10.655 mg. CO_2 .
 $C_{13}H_{16}O_3$. Calculated. C 70.88, H 7.31
Found. " 70.91, " 7.09

0.75 gm. of an acid fraction was recovered and proved to be α -anisylvaleric acid. On fractionation it gave a rough boiling point of 180–185° at 7 mm. and slowly crystallized. The crystalline material melted at 50–52° and was soluble in the usual organic solvents.

4.025 mg. substance: 2.953 mg. H_2O , 10.390 mg. CO_2 .
 $C_{13}H_{16}O_3$. Calculated. C 70.24, H 8.15
Found. " 70.40, " 8.21

Hydrogenation of $\Delta^{\alpha,\beta}$ -Butenolide—This lactone has previously been prepared by Lespieau,¹⁸ and Lespieau's lactone has been nearly quantitatively oxidized to erythronic lactone by Braun.¹⁹ Lespieau's preparation was made by thermal decomposition of 3,4-dichlorobutyric acid. However, 3-chlorocrotonic acid could be readily prepared by the recent method of Braun,²⁰ so $\Delta^{\alpha,\beta}$ -butenolide in this investigation was prepared by thermal decomposition of 3-chlorocrotonic acid as follows:

39 gm. of 3-chlorocrotonic acid were heated for 30 minutes in a metal bath kept at 200–220° under a reflux. A current of dry air was swept through the apparatus during the heating in order to remove HCl gas which was vigorously evolved during the first 10 minutes. The residual brown syrup was dissolved in ether. The solution was washed with sodium bicarbonate solution and dried. Distillation gave 5.8 gm. of butenolide of b.p.₁₃ 94–95° (Lespieau¹⁸ gives b.p.₁₃ 95–96°). The material in dilute solution did not reduce ammoniacal silver nitrate solution at ordinary temperature but upon the addition of dilute NaOH the reduction was prompt. A positive nitroprusside test was obtained.

¹⁸ Lespieau, R., *Bull. Soc. Chim.*, series 3, **33**, 466 (1905).

¹⁹ Braun, G., *J. Am. Chem. Soc.*, **51**, 235 (1929).

²⁰ Braun, G., *J. Am. Chem. Soc.*, **52**, 3167 (1930).

6.785 mg. substance: 3.030 mg. H_2O , 14.190 mg. CO_2 .

$C_6H_8O_2$. Calculated. C 57.14, H 4.76

Found. " 57.04, " 4.99

For the hydrogenation 4.55 gm. of the lactone and 60 mg. of catalyst were used. 1321 cc. of H_2 were absorbed during 70 minutes. Calculated for 1 mol of H_2 , 1214 cc. After filtration from catalyst the solution smelled of butyric acid. Direct titration of free acid required 6.8 cc. of N NaOH, or 13 per cent of acid formation. Saponification with an excess of alkali required an additional 50.0 cc. of N NaOH, or a total of 56.8 cc. Calculated, 54.2 cc.

After relactonization 2.75 gm. of crude butyrolactone were recovered. This boiled at 201° .

5.648 mg. substance: 3.592 mg. H_2O , 11.610 mg. CO_2 .

$C_6H_8O_2$. Calculated. C 55.81, H 6.98

Found. " 56.06, " 7.08

Hydrogenation of β -Phenyl- δ -Caprolactone—This lactone was readily prepared by reduction of β -phenyl- δ -ketocaproic acid in alcoholic solution with sodium. A refluxing solution of 12 gm. of the keto acid in 50 cc. of absolute alcohol was treated with 7.2 gm. of sodium during 20 minutes. During a further 20 minutes 60 cc. of alcohol were added to decompose all of the sodium. After dilution the alcohol was removed under diminished pressure. The residual aqueous solution was relactonized with an excess of H_2SO_4 . The ether extract yielded 4.6 gm. of lactone of b.p.₁₉ $197-200^\circ$.

4.240 mg. substance: 2.950 mg. H_2O , 11.775 mg. CO_2 .

$C_{12}H_{14}O_2$. Calculated. C 75.75, H 7.42

Found. " 75.74, " 7.78

3.2 gm. of the lactone with 0.15 gm. of catalyst absorbed 1222 cc. of H_2 in 70 hours. Calculated for 3 mols of H_2 , 1131 cc. Direct titration required only 0.46 cc. of N NaOH, while saponification by boiling several hours with an excess of alkali required 13.90 cc. of N NaOH, or a total of 14.36 cc. of N NaOH. Calculated, 16.8 cc. of N NaOH.

After relactonization the acid portion recovered was negligible. The crude neutral fraction of β -cyclohexyl- δ -caprolactone weighed

2.9 gm. This material which was unquestionably a mixture of stereoisomers partially crystallized at room temperature. It still gave a mixture of liquid and crystals when distilled, and did not completely crystallize after long standing. This material gave a b.p.₂₀ 194–198°.

8.238 mg. substance: 7.580 mg. H₂O, 22.197 mg. CO₂.

C₁₅H₂₀O₃. Calculated. C 73.39, H 10.29

Found. " 73.49, " 10.31

FURTHER OBSERVATIONS ON THE RELATION OF CALCIUM AND PHOSPHORUS INTAKE TO THE HYPERCALCEMIA AND HYPERPHOSPHATEMIA INDUCED BY IRRADIATED ERGOSTEROL

BY JAMES H. JONES AND MILTON RAPOPORT

(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia)

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In 1922 Zucker, Johnson, and Barnett (1) showed that if a rickets-producing diet were made acid by replacing the calcium lactate of the diet with calcium chloride, only minimal rickets was produced. Similar results were obtained by adding 2 per cent of ammonium chloride to the diet. These authors concluded that the greater acidity increased the solubility of the calcium and phosphorus compounds thus facilitating the absorption of these elements from the intestinal tract. Shortly after this work Orr, Holt, Wilkins, and Boone (2) showed that infants with active rickets failed to retain calcium and phosphorus although adequate amounts were present in the diet. Irradiation of the infants caused large amounts of calcium and phosphorus to be retained with an increased urinary excretion of these elements. They concluded that the ultra-violet radiations in some manner enhanced the absorption of calcium and phosphorus from the intestines.

In a later publication Zucker and Matzner (3) reported that the feces of rachitic rats were more alkaline than those of controls, and the administration of cod liver oil caused the pH of the feces of rachitic rats to change from the alkaline to the acidic side of neutrality. These results have been amply confirmed, but Shohl and Bing (4) have demonstrated that rickets produced on the high calcium, low phosphorus diet of Steenbock and Black (5) can be cured by the addition of alkaline phosphate, and furthermore, if this diet is irradiated it is no longer rachitogenic, but the feces do not become acidic in reaction.

The more recent work on irradiated ergosterol also has indicated that the antirachitic factor can in some way or other increase the absorption of calcium from the intestinal tract or decrease its elimination by this path. Thus Bills and Wirick (6), Shohl, Goldblatt, and Brown (7), and Harris and Innes (8) have demonstrated that the degree of toxicity induced by large doses of irradiated ergosterol, especially the extent of abnormal calcification of tissues, is somewhat dependent upon the amount of calcium and phosphorus in the diet. It appears that on diets low in calcium it is practically impossible to produce extensive calcification of tissues regardless of the quantity of irradiated ergosterol given. In a previous publication by us (9) more direct proof was presented that the net absorption of calcium from the intestinal tract is increased by irradiated ergosterol. It was shown that certain doses of irradiated ergosterol produced a marked hypercalcemia if the diet contained calcium but not if this element were omitted from the diet.

As a continuation of our earlier work (9) on the influence of the amount of calcium in the diet on the hypercalcemia induced by overdosage of irradiated ergosterol, we have extended the experiments to include a study of the results obtained when much larger doses of irradiated ergosterol are given. The question of the influence of vitamin D on intestinal absorption has also been attacked by a different method.

Although there may be two factors in irradiated ergosterol (one vitamin D and another which produces hypercalcemia and abnormal calcification) as claimed by Holtz and Schreiber (10) the two factors must be closely related and both influence calcium metabolism; consequently, in this paper they are treated as a single substance.

EXPERIMENTAL

The plan of the first experiments reported here is the same as that previously used (9), *viz.* dogs were given a diet extremely low in calcium for several days, followed by a period of irradiated ergosterol administration, and the changes in the level of the calcium of the blood were followed during this time. The ergosterol preparation used was furnished by Mead Johnson and Company and according to their standardization had an antirachitic potency

of 10,000 X or 30,000 D. It is not necessary to give the details of these experiments for invariably when 0.15 cc. of this preparation was given daily per kilo of body weight, a marked hypercalcemia resulted within a few days. Dog 84, as an example, had a calcium level of 12.2 mg. per 100 cc. of serum at the beginning of the administration of irradiated ergosterol, and 7 days later the calcium of the serum had risen to 15.93 mg per cent.

It was also found possible to produce an increase in the level of blood calcium in thyroparathyroidectomized dogs being fed on the calcium-free diet if equally large doses of the vitamin D preparation were given. The calcium in the serum of Dog 93 (6.4 kilos in weight) dropped from 11.50 mg. per 100 cc. at the time of the removal of the parathyroids to 8.31 mg. 4 days later. At this time the daily administration of 1 cc. of the irradiated ergosterol solution was begun, and 12 days later the calcium had risen to 19.66 mg. per 100 cc. of serum. This confirms our previous results (11) which showed that it is possible by the administration of large doses of irradiated ergosterol to control the tetany in dogs resulting from parathyroidectomy.

Previously we reported that the only symptom accompanying the hypercalcemia in dogs, induced by overdosage of vitamin D, was the loss of appetite. In the experiments from which those results were obtained comparatively small overdoses of irradiated ergosterol had been given. In the experiments reported here much larger amounts of vitamin D were employed and the results were quite different. Within a few days the animals became very quiet, lost weight rapidly, and several of them died within 2 weeks after the feeding of irradiated ergosterol was begun. One dog had a grossly visible hematuria. At autopsy the dogs showed marked hemorrhagic gastritis and enteritis, and in some cases the stomach was distended with a bloody liquid. No abnormal calcifications were discernible by macroscopic examination.

These data when considered with those previously published indicate that irradiated ergosterol, at least when given in large amounts, can mobilize calcium from the intestinal tract and also from stores within the body. The former action, however, appears to be the more pronounced and can be demonstrated with smaller doses of vitamin D than the latter. Harris and Innes (8) arrived at similar conclusions from histological studies of rats

given graded doses of irradiated ergosterol in addition to diets containing varying amounts of calcium.

We have already shown (9) that irradiated ergosterol in amounts which produce a marked hypercalcemia in dogs on a diet containing calcium may have little influence on the level of blood calcium when given in addition to a calcium-free diet. If, however, after the calcium-free diet and irradiated ergosterol have been given for 2 or 3 weeks, a liberal quantity of a soluble calcium salt is given by mouth, a marked hypercalcemia results within 20 hours. It was thought that a more detailed study of this latter observation might furnish additional information concerning the mode of action of irradiated ergosterol. Warkany (12) has already made a detailed study of the influence of irradiated ergosterol upon the absorption of phosphorus from the intestines of rabbits. He gave to normal rabbits by mouth 0.5 gm. of disodium phosphate per kilo of body weight. At the time of phosphate administration, and every 2 hours thereafter for 8 hours, samples of blood were taken from the animals and the sera were analyzed for inorganic phosphates. The rabbits were then each given a single large dose of irradiated ergosterol, and 48 hours later a quantity of disodium phosphate similar to that originally given was administered, and the blood sampling was done as before. The extent of the hyperphosphatemia produced by equal amounts of soluble phosphate before and after giving irradiated ergosterol could thus be compared on the same animal. Warkany found that there was an increase in the level of serum phosphates both before and after ergosterol administration. The extent of the hyperphosphatemia after giving vitamin D, however, was considerably greater than before vitamin D was administered. The average maximum rise before was 1.7 mg. and after 5.2 mg. per 100 cc. of serum, but in both instances the hyperphosphatemia were of the same duration. The excretion of phosphorus was considerably increased following irradiated ergosterol. From these results Warkany concluded that the rise in concentration of blood phosphorus was the result of increased absorption and not decreased elimination.

In our experiments dogs were used; otherwise the general procedure was very similar to that described by Warkany. In addition to giving disodium phosphate, calcium gluconate and dicalcium phosphate were each studied in a similar manner, and in each

case the calcium level of the blood was followed as well as that of the inorganic phosphate.

A simple diet composed of 99 parts of whole yellow corn and 1 part of sodium chloride was used as the basal ration. To the corn and salt sufficient water was added to make a soft mash and the mixture was cooked in a steam autoclave at 1 atmosphere pressure for about 2 hours. This diet contains very little phosphorus and still less calcium (0.51 per cent CaO). Watchorn (13) as well as Harris and Innes (8) has used the Steenbock and Black (5) rickets-producing diet, from which the calcium carbonate had been omitted, as a basal diet for various calcium studies. They claim it to be free of calcium, but we have never found a sample of either yellow corn or wheat gluten to be entirely free of calcium. Elmslie and Steenbock (14) state that a somewhat similar diet containing 77 per cent yellow corn and 20 per cent wheat gluten had a CaO content of 0.45 per cent which coincides very closely with our analyses. Since the wheat gluten was found to contain more calcium than the corn the former was omitted from the ration.

In all the experiments about 20 cc. of blood were taken at each bleeding by heart puncture. The calcium analyses were made on the sera in duplicate by the method of Clark and Collip (15). Single determinations of the inorganic phosphorus of the sera were carried out by the Fiske and Subbarow (16) method. The irradiated ergosterol solution which usually was given about 1 week after the first series of bleedings had been completed was administered directly from a pipette, and the dose of 0.5 cc. per kilo of body weight was kept constant throughout. The various salts were administered in aqueous solution or suspension by stomach tube and were given approximately 48 hours after the vitamin D preparation had been fed. A few of the animals were used for more than one experiment, in which cases the dogs were given a rest period of about 4 weeks between consecutive experiments, during which time they were fed a mixed table scrap diet but were again returned to the basal diet several days before another series of observations was begun.

The results of these experiments are summarized in the accompanying curves (Charts I to III). One control dog was given water by stomach tube and bled five times at 2 hour intervals.

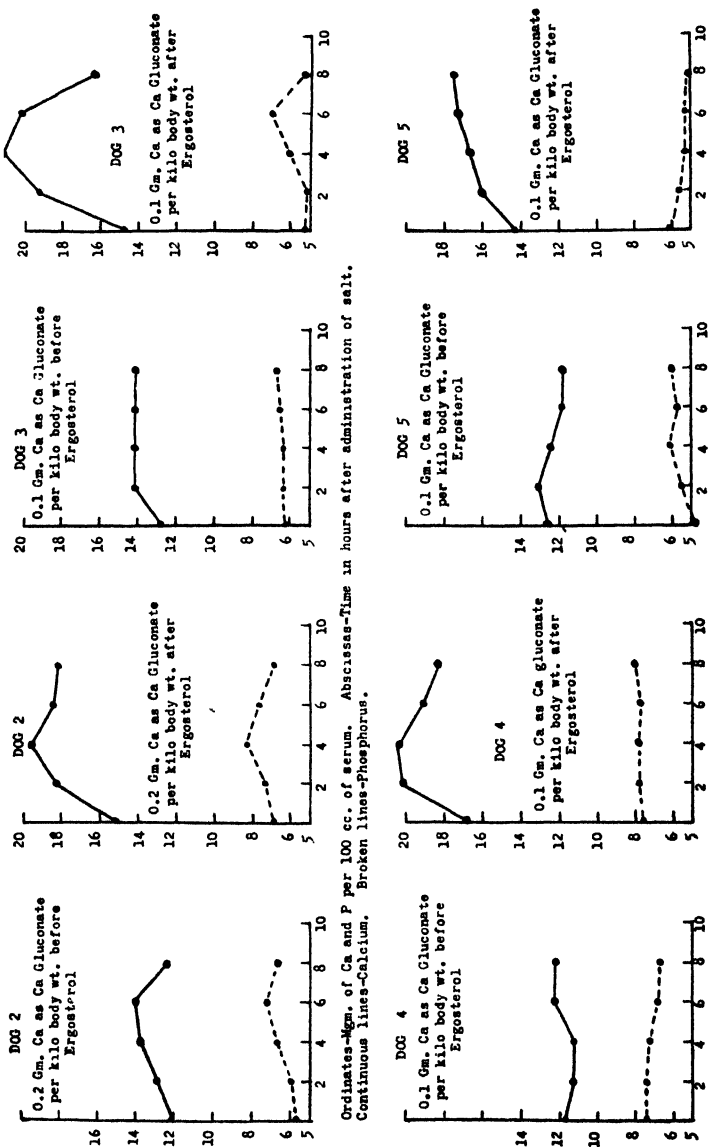


CHART I. Comparison of the effect of feeding calcium gluconate on the concentrations of serum calcium and phosphorus before and after the administration of a single large dose of irradiated ergosterol.

A few days later this same dog was given a dose of irradiated ergosterol and after 48 hours had elapsed water was again given by stomach tube and the blood sampling was carried out as before. The bleedings had no effect on the concentration of phosphorus or calcium either before or after the administration of the vitamin D preparation. Hjort (17) has previously shown that a series of bleedings has no particular influence on the concentration of calcium in the serum.

In Chart I are to be found the results of the experiments conducted with calcium gluconate. Dog 2 was given 0.2 gm. of calcium as calcium gluconate per kilo of body weight both before and after irradiated ergosterol administration. In both cases there was an increase in the concentration of blood calcium for the first few hours following the administration of the calcium salt, but the rise was more pronounced after vitamin D had been given than before. Dogs 3, 4, and 5 were given but 0.1 gm. of calcium per kilo of body weight. The amount was reduced in order to minimize, if possible, the hypercalcemia resulting from giving the salt before irradiated ergosterol administration. As seen from the charts, when this amount of calcium was given before vitamin D feeding there was a very slight increase of calcium in the blood, whereas a marked rise invariably resulted when the salt was given following the irradiated ergosterol. The average increase in the former was 1.12 mg. and in the latter 4.24 mg. per 100 cc. of serum.

Another important observation was that these four dogs without exception vomited within 30 to 45 minutes following the second feeding of the calcium salts, that is, the feeding after irradiated ergosterol had been given. No vomiting ever occurred when an equal quantity of calcium gluconate was given before vitamin D administration. Not only did the dogs vomit but a complete change of appearance came over them in this short period. At the time of giving the salt the dogs were always active and playful and remained so for a few minutes. Gradually they became less playful but very restless, and their eyes became dull in appearance. These symptoms were soon followed by vomiting. In one case the vomitus was tested qualitatively and found to contain an abundance of calcium. In spite of this loss of calcium a greater hypercalcemia was produced in these cases than in those where no irradiated ergosterol had been given even though all of the calcium had been retained by the animal.

As seen from the curves the giving of calcium produced slight hyperphosphatemia in some cases, whereas in others there was no noticeable effect. The degree of hyperphosphatemia seems to bear no relationship to the extent of increase in blood calcium.

After the experiments on calcium similar studies were made on the feeding of phosphates. The results are given in Chart II, Dogs 4, 6, and 7. Considering first the influence of these procedures on the level of blood phosphorus, it is seen that even when no irradiated ergosterol had been given a marked hyperphosphatemia followed the feeding of liberal quantities of disodium phosphate. Dog 6 received 0.1 gm. of phosphorus per kilo of body weight as disodium phosphate. Both before and after irradiated ergosterol administration there was a decided increase in the concentration of blood phosphorus. The increase was, however, somewhat greater after vitamin D feeding than before. Dog 4 was given 0.05 gm. of phosphorus per kilo of body weight which also produced a rather pronounced increase in the concentration of blood phosphorus and which was not materially increased by the irradiated ergosterol.

Dog 7 like Dog 4 was given 0.05 gm. of phosphorus per kilo of body weight. In this case, as with Dog 6, there was a decided difference between the hyperphosphatemia produced before and after irradiated ergosterol administration. The duration of the hyperphosphatemia in each case was very short and the return to normal was very abrupt. The average rise in concentration of blood phosphate of these three animals was 3.84 mg. per 100 cc. of serum before and 5.48 mg. after the administration of the vitamin D preparation.

The influence that feeding disodium phosphate had on the concentration of blood calcium is of interest although it gives no information concerning the action of irradiated ergosterol. Binger (18) has shown that if soluble phosphates, either neutral or alkaline, are injected into an animal intravenously there is a marked diminution in the concentration of serum calcium. These results have been repeatedly verified, but as far as the authors have been able to find, no report has been made that similar results have been obtained by oral administration of phosphates. However, the calcium curves of Chart II show very definitely that a marked hypocalcemia is produced when large amounts of di-

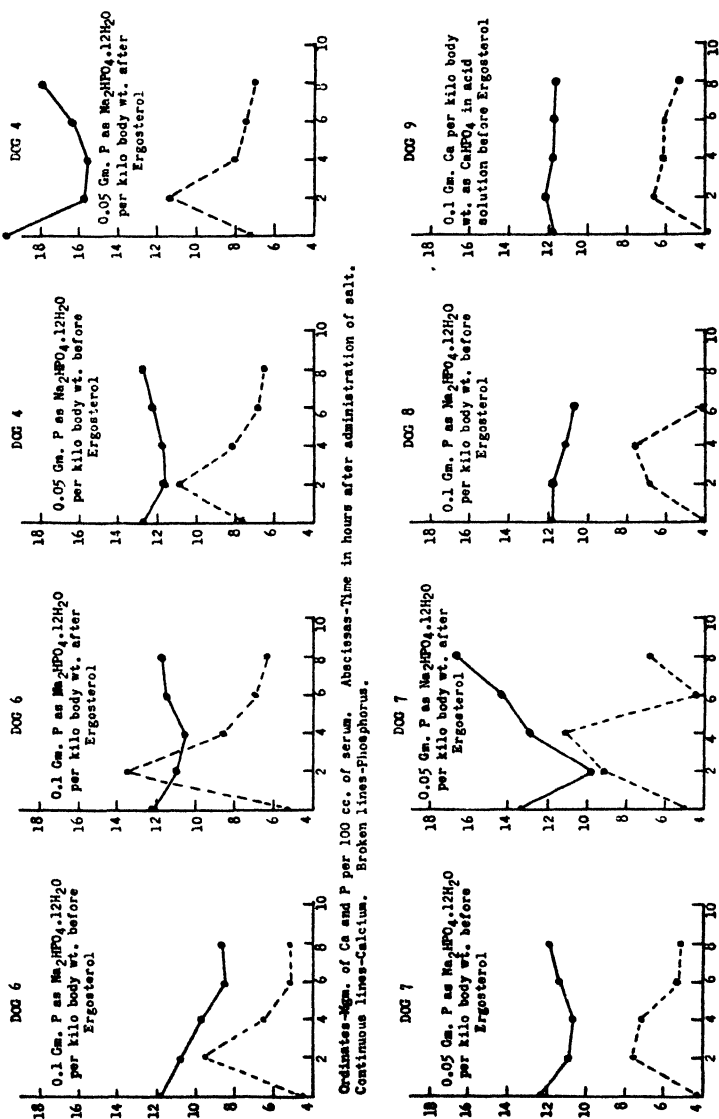


CHART II. Comparison of the effect of feeding disodium phosphate on the concentrations of serum calcium and phosphorus before and after the administration of a single large dose of irradiated ergosterol.

sodium phosphate are given *per os*. The drop in calcium occurred in all the dogs which received the phosphate both before and after irradiated ergosterol. The general tendency of the calcium curve in these cases was the opposite of the phosphorus curve. That is, as the phosphorus increased in concentration the calcium decreased, and as the phosphorus returned to normal the calcium did likewise. In the first phosphate feeding of Dog 6 the calcium remained low although the phosphorus had returned to normal at the end of the bleeding period. Dog 7 also showed an exception in that following the second phosphate feeding the calcium not only returned to the level preceding the administration of phosphate, but it rose to several mg. above the original value. The results obtained with Dog 8 are also included in Chart II simply as another example of the influence of phosphate on the level of serum calcium. This dog died after the first half of the experiment was completed and before irradiated ergosterol was given.

The next step in the investigation was to study the results of feeding calcium and phosphorus at the same time. The first attempts along this line were made with an aqueous suspension of dicalcium phosphate to which sufficient hydrochloric acid was added to effect solution. It was soon found, however, that such a solution was too acid to be retained by the animal. In contrast to the vomiting described above which occurred from 30 to 45 minutes after feeding calcium gluconate, the animals invariably vomited within 10 minutes after the acid solution of calcium phosphate had been given. One dog (Dog 9, Chart II) did retain the solution. It is of interest because there was a rather marked increase in the concentration of serum phosphates without an accompanying drop in calcium. Whether this is due to the presence of calcium or the increased acidity cannot be said, but it agrees with the results of Binger who found that no hypocalcemia resulted if a phosphate solution with a pH below 6 were injected.

Since it seemed impossible to give a solution of calcium phosphate the dibasic salt was suspended in water and given by stomach tube. The results are summarized in Chart III. Dogs 10 and 11 showed slight increases in phosphorus and decreases in calcium before irradiated ergosterol was given. After administration of vitamin D the calcium of Dog 10 remained constant with a very slight rise in phosphorus, and Dog 11 showed a slight rise in

calcium with the phosphorus remaining constant. The phosphorus of Dog 4 rose slightly, the calcium remained practically stationary before irradiated ergosterol, while the phosphorus rose slightly with the production of a marked hypercalcemia when the salt was given following vitamin D administration. None of these dogs gave any evidence, such as vomiting, that the dicalcium

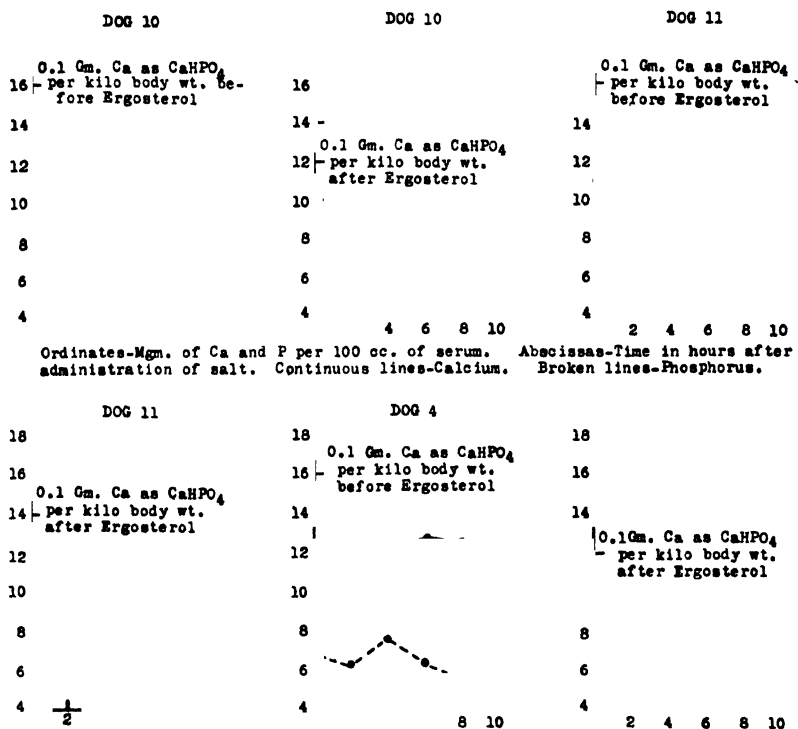


CHART III. Comparison of the effect of feeding dicalcium phosphate on the concentrations of serum calcium and phosphorus before and after the administration of a single large dose of irradiated ergosterol.

phosphate caused any distress as was found when calcium gluconate was given after irradiated ergosterol.

Because of the variation in the results with dicalcium phosphate no definite conclusions can be drawn, but it is clear that irradiated ergosterol does not increase absorption of calcium and phosphorus when these two elements are given together and in an insoluble

form to the extent it does when each is given separately and in solution. The results obtained with Dog 4 are interesting in view of the fact that there was a marked increase in the concentration of calcium but not of phosphorus.

The results of these experiments give very strong support to the view that vitamin D functions, at least in part, by increasing the absorption of calcium and possibly phosphorus from the intestinal tract. The data obtained from the study of the phosphate feeding are not as conclusive as the results of the calcium experiments. One reason is the rather marked hyperphosphatemia produced by phosphate feeding before the administration of irradiated ergosterol. This result is somewhat different from that obtained by Warkany who found that only a slight increase in serum phosphorus resulted from feeding even larger doses of disodium phosphate than we used. His results also differ from ours in that the irradiated ergosterol increased the level of serum phosphorus even before the phosphate was administered, whereas it had no influence upon the level of calcium. In our experiments we found that the calcium was more frequently increased by the action of irradiated ergosterol *per se* than the phosphorus. These differences, however, may be due to the fact that Warkany used rabbits instead of dogs, that the basal diets were different, and that the amounts of activated ergosterol administered were not the same.

No light has been thrown upon the mechanism by which vitamin D increases the absorption of calcium and possibly phosphorus from the intestines. Some doubt, however, may be cast upon the theory that it is the result of an increased solubility of these elements. If it were a simple question of solubility one would expect that a calcium salt already in solution would be absorbed as readily in the absence of vitamin D as in its presence, especially on a diet low in phosphorus. If precipitation takes place due to the alkalinity of the intestinal contents the calcium must unite with some radical other than the phosphate. The radical may be the carbonate which would naturally be present in the intestines. If vitamin D does increase solubility it would seem that calcium administered in the form of dicalcium phosphate in the presence of a large excess of vitamin D would be absorbed as readily as if the calcium were given in a soluble form such as the gluconate.

Bauer, Marble, Maddock, and Wood (19) have concluded from studies on humans that irradiated ergosterol tends to decrease the free acidity of gastric juice and to increase the alkalinity of the pancreatic secretion, which is somewhat of a contradiction of the early theories that the intestinal contents become more acid under the influence of vitamin D. Further work is necessary to explain the mechanism whereby vitamin D increases the rate of absorption of calcium and possibly phosphorus from the intestinal tract.

SUMMARY

Dogs given 0.15 cc. of a solution of irradiated ergosterol (30,000 D) per kilo of body weight per day developed a hypercalcemia in a few days even though there was no calcium in the diet. Other symptoms such as rapid loss in weight accompanied the hypercalcemia and at autopsy the animals frequently showed hemorrhagic gastritis and enteritis. The above large dose of irradiated ergosterol protected dogs on the calcium-free diet against parathyroid tetany.

If 0.1 gm. of calcium per kilo of body weight in the form of calcium gluconate were given to a normal dog by stomach tube and the blood serum analyzed every 2 hours (for 8 hours) for calcium and phosphorus, it was found that there was a slight rise in calcium and sometimes a slight rise in phosphorus. If 0.5 cc. of irradiated ergosterol solution (30,000 D) per kilo of body weight were given to the animal and 48 hours later the calcium salt again administered, the rise in concentration of serum calcium was much more pronounced than before the irradiated ergosterol was given. The increase in the concentration of phosphorus was of about the same order as before vitamin D administration.

Similar experiments with disodium phosphate (0.05 gm. of P per kilo of body weight) produced rather marked hyperphosphatemia both before and after administration of irradiated ergosterol. The latter, however, was of slightly greater magnitude than the former. The phosphate invariably caused the concentration of serum calcium to fall both before and after irradiated ergosterol.

If calcium and phosphorus were given together as an aqueous suspension of dicalcium phosphate (0.1 gm. of Ca per kilo of body weight) there was a slight fall in the concentration of calcium and

practically no change in the concentration of phosphorus before irradiated ergosterol, whereas after the administration of irradiated ergosterol there was an increase in the level of calcium with the phosphorus level remaining approximately constant. The hypercalcemia, however, was not as great as that produced by calcium gluconate following administration of the vitamin D solution.

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CHANGE OF ROTATORY POWER OF PURIFIED EGG ALBUMIN AS EVIDENCE OF THE MODE OF COMBINATION OF ACID AND ALKALI WITH PROTEINS

BY H. J. ALMQUIST AND DAVID M. GREENBERG

(From the Division of Biochemistry, University of California Medical School, Berkeley)

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During the course of investigative work on natural egg white conducted by one of the writers it was noticed that the rotatory power of the proteins changed through a wide range on the addition of varying amounts of alkali. Since this effect gave promise of affording a means of obtaining direct evidence on the type of combination of proteins with acid and alkali, it was considered desirable to carry out measurements on a purified individual protein. Accordingly egg albumin, which is one of the best characterized of proteins, was chosen, and measurements were made on the influence of additions of acid and alkali on the optical rotation.

The egg albumin was prepared from fresh egg white according to the method of Sørensen (1) as modified by La Rosa (2) and freed from ammonium sulfate and other crystalloids by dialysis. In this way there was prepared a stock solution containing 6.97 per cent albumin practically free from ash and having a pH of 5.04. A 25 cc. portion of the stock solution was placed in a 50 cc. volumetric flask, the desired amount of acid or alkali added, and the mixture made up to a volume of 50 cc. The rotatory power of the solution was determined in a 20 cm. tube, the average of five readings on a Schmidt and Haensch polarimeter at the D line of sodium being used. pH values, by the hydrogen electrode, were determined on at least three separate portions and the average value taken.

The specific rotation, $[\alpha]_D^{25}$, of the egg albumin was found to be -30.8° at the pH of the purified solution. This value was altered

but very little until the addition of sodium hydroxide had brought the pH to 11.00. From pH 11.00 to 12.60 the rotatory power changed sharply with added increments of base, reaching a definite maximum of -60.6° . With the addition of strong acid the rotatory power changed in the same direction as with alkali, going rply from the value of -30.8° in the isoelectric region to a

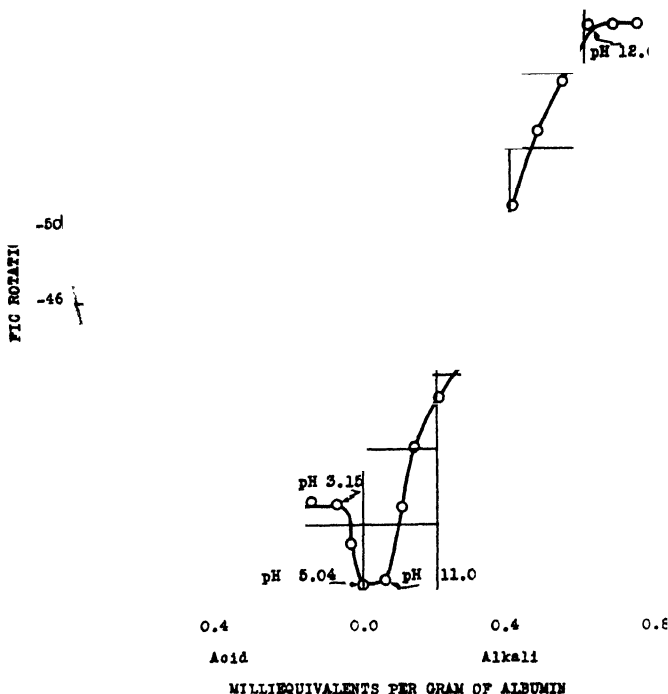


FIG. 1. The change of rotatory power of purified egg albumin with addition of acid and alkali.

value of -38° at a pH of 3.15 and remaining constant at this value to a pH of 1.72, beyond which further measurements were not made. On addition of either acid or alkali the optical rotation at once attained a constant value and could be immediately restored to the original value by the addition of an equivalent amount of alkali or acid. The rotation remained unchanged during several hours in certain of the mixtures which were saved for

this test. The data are plotted in the form of milli-equivalents of acid or alkali added per gm. of albumin as abscissa and specific rotation as ordinate in Fig. 1. The pH points of particular interest are marked with arrows.

Although this type of alteration of optical properties has apparently not been described in the literature, a precedent for this behavior is to be found in data reported for certain amino acids, as summarized by Mitchell and Hamilton (3). Considerable variation of specific rotation is obtained on the addition of acid or alkali to solutions of the amino acids, the changes in both cases being in the same direction. The maximum values of specific rotation are regarded as expressing the optical activities of the salts of the amino acids. The behavior of purified egg albumin appears to be analogous to that of the amino acids and may logically be explained on the same basis.

At the high alkalinity of pH 12.6, where the rotation maximum is reached with sodium hydroxide, the albumin has attained its maximum base combination according to the summarized results given by Cohn (4) and our own figures. On the other hand, in the acid region maximum rotation is already attained at the pH of 3.15, which is quite far from the region of maximum acid combination (4). This points to the change of rotation of the protein with acid being quite complex and not to be attributed to ionization of the protein alone. The instantaneous change of rotation on the addition of acid or alkali, the ready reversibility of the change, and the well defined maxima indicate rapid interaction between protein and reagent ions and typical salt formation. The effect is not to be ascribed to denaturation of the egg albumin which occurs in the extreme pH regions but which requires a considerable length of time. The magnitude of the changes, almost a doubling of the specific rotation with the alkali, is such as to preclude explanations based on adsorption theories of combination.

A noteworthy feature of the present results is the change in the pH region 11.00 to 12.60 in which the variation of rotation with alkali occurs. While certain other workers, more prominently Hoffman and Gortner (5), have insisted that the binding of alkali by protein in high pH regions is predominantly colloidal adsorption, the evidence secured by the study of optical activity points strongly toward extensive combination of a chemical nature.

This, it may be pointed out, is in harmony with the findings of Rawlins and Schmidt (6) by the method of dye titration. The work briefly described here is to be continued.

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AN APPLICATION OF THE URANYL ZINC ACETATE METHOD FOR DETERMINATION OF SODIUM IN BIOLOGICAL MATERIAL

BY A. M. BUTLER AND ELIZABETH TUTHILL

(From the Infants' and Children's Hospitals and the Department of Pediatrics, Harvard Medical School, Boston)

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INTRODUCTION

In 1928, Barber and Kolthoff (1) described a method for quantitatively precipitating sodium with uranyl zinc acetate and gravimetrically determining the sodium as uranyl zinc sodium acetate, $(\text{UO}_2)_2\text{ZnNa}(\text{CH}_3\text{COO}) \cdot 6\text{H}_2\text{O}$. Samples with 2 mg. of sodium gave accurate results. In this paper and a subsequent one (2), they discussed the ions interfering with the determination. Potassium interfered if the solution contained more than 25 mg. per cc. as the chloride or less as the sulfate. Phosphate was precipitated by the reagent, thus interfering with the determination of sodium, and had to be removed first.

The method has been tried for analysis of biological material in several laboratories. In each instance the phosphate has been precipitated first as uranyl phosphate. In our hands this method of removing phosphate has not been satisfactory. The uranyl phosphate is a slimy precipitate and the results for sodium are too low. Barber and Kolthoff reported the same difficulty. They obtained satisfactory results with known salt solutions after removing phosphate by precipitation with magnesia mixture.

As the determination of Na in biological work is of importance and the methods at hand are either laborious, expensive, or subject to considerable error, we studied the applicability of the Barber-Kolthoff method to biological material. The information obtained and modifications introduced have resulted in the development of definite procedures for the determination of sodium in urine, serum, and material high in phosphate and potassium such as

whole blood, stool, and tissue. Powdered $\text{Ca}(\text{OH})_2$ was used as the means of precipitating phosphate and found to be satisfactory, within the desired limits of error, and more convenient than the magnesia mixture. It simplifies the reagents required for the procedure. Its addition clarifies cloudy urines and produces no volume change, thus eliminating the necessity of diluting to known volume and the taking of aliquots, or the quantitative washing of the precipitate. For urines containing protein we again have resorted to a solid precipitating reagent, HgCl_2 , and so again have avoided volume changes. By precipitating the triple sodium salt in a stoppered glass filter, we have eliminated the necessity of washing the precipitate to the filter, thus saving time and reagent. We have studied the necessity of ashing urines and have investigated conditions for determining sodium in urines containing relatively very small amounts of this element.

Determination of Na in Urine

Reagents

1. *Uranium Zinc Acetate Reagent*—Solution A consisted of 80 gm. of Na-free uranium acetate, $\text{UO}_2(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 2\text{H}_2\text{O}$, and 48 gm. or 46 cc. of 30 per cent acetic acid (per cent by volume), plus water to make 520 gm. Solution B consisted of 220 gm. of zinc acetate, $\text{Zn}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 2\text{H}_2\text{O}$, and 24 gm. or 23 cc. of 30 per cent acetic acid, plus water to make 520 gm.

Cover and warm both solutions on a steam bath until, with stirring, solution is complete. Mix while hot and let stand for 24 hours before using. If no yellow precipitate appears add 0.2 gm. of precipitated uranyl zinc sodium acetate in order to saturate with this triple salt. Shake the mixture several times before using and filter to assure saturation at the temperature of the analysis.

2. *Phenolphthalein*—1 per cent alcoholic solution.

3. *Powdered HgCl_2* .

4. *Powdered $\text{Ca}(\text{OH})_2$* .

5. *Saturated Solution of NH_4ClO_4*

6. *95 Per Cent Alcohol Saturated with Precipitated Uranyl Sodium Zinc Acetate.*

A. Procedure for Urines of Ordinary Na Content

Measure into a small flask approximately 6 cc. of urine. Add 1 drop of phenolphthalein and 0.2 gm. of powdered calcium hydroxide.

(If the urine contains protein take 10 cc. instead of 6 and add 0.05 gm. of HgCl_2 . Then add the phthalein and calcium hydroxide.) Shake and let stand for 30 minutes with occasional shaking. The solution should turn pink. Filter through a fine filter paper and collect the filtrate in a test-tube. (If the urine contains protein, test the filtrate for protein. If protein is still present add more HgCl_2 and refilter.) Stopper the test-tube in order to prevent precipitation of CaCO_3 . Fit a solid rubber stopper from below into the bottom of a 30 cc. capacity porous glass filter¹ that has been dried and accurately weighed.² The filter now stands on the rubber stopper. The stopper prevents the liquid from going through the filter even though there is considerable air space between stopper and porous glass. Pipette approximately 20 cc. of freshly filtered reagent to the filter. The reagent should be shaken frequently preceding use to insure saturation. Pipette 2 cc. of urine filtrate directly to the reagent in the filter.³ If the pipette is graduated for blow-out delivery and one makes the delivery by blowing instead of warming by hand, a precipitation of CaCO_3 may occur. Aside from dirtying the pipette, this does not matter as this precipitate redissolves immediately. Stir with a small glass rod till a precipitate appears, and several minutes thereafter. Stirring is particularly important where there is a small amount of Na present. Under such conditions insufficient stirring will give results from 2 to 5 per cent too low. Withdraw the stirring rod, rinsing it with 3 to 5 cc. of reagent as it is withdrawn. Cover the filter with a watch-glass and let it stand at approximately constant room temperature for 1 hour. Remove the rubber stopper, place the filter in a suction flask, and apply suction. After the reagent has been filtered off, wash the precipitate with five 2 cc. portions of 95 per cent alcohol saturated with the triple salt. The alcohol reagent should be filtered before use. Care should be taken to wash down the sides of the filter. Delaying the washing makes this more difficult. Finally wash with two 5 cc. portions of ether.⁴

¹ Jena glass filters Size 2, capacity 30 ml., porosity 1 G4, may be obtained from the Empire Laboratory Supply Company.

² Clean and dry with alcohol and ether, applying suction, and let stand for $\frac{1}{2}$ hour in a desiccator.

³ When urines are low in Na see modified procedure under (B).

⁴ As might be expected from the character of the reagents used, some salt adheres to the precipitate. When washing is carried out as directed the gain in weight is, however, only from 0.2 to 0.5 mg. The accuracy of the determination being considered, this error is small.

Continue suction until the precipitate is thoroughly dry, place in a desiccator over calcium chloride, and weigh after $\frac{1}{2}$ hour. The precipitate is placed in a desiccator to prevent its taking up water while coming to the temperature of the balance. As no change in weight occurs, longer standing is unnecessary.

Run a blank on an equal volume of distilled water and reagents.

The ratio of volume of urine to the volume of reagent must not be greater than 1:10. As the safe capacity of the filter is 25 cc., the largest urine filtrate that can be pipetted directly to the filter is 2.5 cc. 25 cc. of the reagent will precipitate 20 mg. of Na. The likelihood of urines exceeding 20 mg. per 2 cc. is very slight. But because of the difficulty of washing large precipitates, it is better not to exceed a precipitate of about 700 mg. Since 1 mg. of Na gives a precipitate of 66.9 mg., the sample of urine taken for analysis should preferably not contain more than 10 mg. of sodium; and when possible not less than 1 mg.

Experience has shown that ashing of the urine is unnecessary, unless large samples are used. Protein must be removed or it will be precipitated by the uranyl reagent.

Calculations

The precipitate contains 1.495 per cent of Na. Hence the weight of Na in the sample is found by multiplying the weight of precipitate, corrected for the blank, by 0.01495. The result is multiplied by $\frac{100}{s}$ to calculate weight of Na per 100 cc. of urine. (s = cc. of urine represented in the sample precipitated.)

$$\text{Gm. Na per 100 cc. urine} = \frac{1.495 (\text{gm. precipitate} - \text{gm blank})}{s}$$

$$\text{M-eq. Na per liter urine} = \frac{650 (\text{gm. precipitate} - \text{gm. blank})}{s}$$

B. Procedure for Urines Low in Sodium

Urines produced on low salt diets or as the result of certain types of diuresis may contain less than 1 mg. of Na per 2 cc. When a urine contains between 0.5 and 0.1 mg. of Na per cc., a volume of urine filtrate containing between 1 and 3 mg. of Na, but not exceeding 10 cc., is pipetted to a small evaporating dish or beaker. 1 drop at a time of concentrated HCl is added until the urine turns

acid. This lessens the formation of precipitate during evaporation. The sample is evaporated down to approximately 2 cc. If it is evaporated down to dryness, 2 cc. of water are added. It is then transferred quantitatively to the reagent in the weighed filter as described for Procedure A, except that approximately 15 cc. of reagent are placed in the filter instead of 20 cc. The beaker is rinsed with 0.5 cc. of water and then with two 3 cc. portions of reagent. The solution in the filter is stirred the proper length of time and the stirring rod rinsed during removal with 5 cc. of reagent. The procedure is then continued as outlined above.

Should the preliminary evaporation of the urine continue to dryness or cause a precipitate to crystallize out, the transfer to reagent in the filter is made as usual, all the precipitate being washed to the reagent. Here the volume and acidity are such as to dissolve this residue. The stirring is continued a little longer in order to assure complete solution.

When the volume of urine used necessitates this concentration procedure and a slimy residue remains after evaporation, one may add 5 cc. of concentrated HNO_3 plus 2 cc. of superoxol, cover, destroy organic matter by heating, and then evaporate to dryness. This will facilitate the transfer but does not seem necessary, as we have incurred no demonstrable error in transferring such residues directly to the reagent in the glass filters.

C. Procedure for Urines of Minimal Sodium Content

In certain experiments carried out in this laboratory where patients were on a very low sodium diet, we have wished to analyze specimens so low in sodium that 40 cc. samples of urine were required in order to obtain weighable precipitates. Such samples evaporated to 2.5 cc. for transfer to reagent have exceeded the permissible potassium concentration. We have, therefore, been forced to remove potassium from these samples and have removed it as precipitated KClO_4 by the following procedure.

Ash 50 cc. of urine by the wet nitric acid plus superoxol method. (In our hands the Stolte method of ashing has been unsatisfactory.) Extract the residue with warm water. Without filtering, transfer the residue quantitatively to a 25 cc. volumetric flask and make up to volume. Mix well and then pour most of the solution into a small Erlenmeyer flask. Add about 0.4 gm. of solid Ca(OH)_2 .

Let this stand for about 1 hour with occasional shaking. Filter and if there is any doubt that the phosphate is completely precipitated, test the first portion of filtrate. (Several times we have been surprised to find phosphate.) From the filtrate transfer 20 cc. to a small beaker and evaporate on a steam bath to dryness. Add 5 cc. of hot water and 1 drop of concentrated HCl; shake for a few minutes and then add 3 cc. of saturated ammonium perchlorate solution. Cool and let stand with occasional shaking for $\frac{1}{2}$ hour while the KClO_4 precipitates. Filter into a beaker and wash quantitatively with 95 per cent alcohol. Evaporate the filtrate to dryness and transfer the entire contents to the uranium zinc acetate reagent in a glass filter as outlined previously, using 2 cc. of water for solution and transfer and 0.5 cc. for the first washing of the beaker. As the undissolved residue is fairly large, it is wise to use several 3 cc. portions of the uranium zinc acetate reagent to complete the transfer and rinsing of the beaker. In this instance the stirring of the solution in the filter must be continued longer in order to assure complete solution of the residue. Let the rod remain in the solution in the filter for about 10 minutes, then stir again and remove the rod with appropriate rinsing. By so doing we have never had any indication of the transferred residue remaining undissolved in the filter. The washing and weighing is carried out as usual. In this instance the sample of urine analyzed would be 40 cc.

In using these larger quantities of urine, ashing is necessary. At first we thought it would be necessary to ash the filtrate from the potassium precipitation in order to get rid of the excess perchlorate. This was found to be unnecessary, and most fortunately so, as the ashing of ammonium perchlorate without spattering is very difficult. We also tried precipitating the potassium with ammonium acid tartrate. This, however, necessitated ashing in order to eliminate the tartrate, which gives a precipitate with the uranium reagent. The method as given seems quite satisfactory and not laborious.

Stool and Tissue

The material is prepared for analysis by evaporating to dryness, powdering, dry ashing, and extracting with dilute HCl (3). The actual determination of Na is made exactly as for urines except that

particular attention should be paid to the complete precipitation of all the PO_4 .

We have not made any analyses of tissue but purposely made the known salt solutions used in Tables I and II similar to the concentration of the inorganic elements found in muscle juice. Should the tissue be wet-ashed with H_2SO_4 it might be necessary to evaporate the sample to dryness and heat sufficiently to drive off the excess H_2SO_4 . As this procedure is easily done without spattering, it should present no difficulties.

Determination of Na in Serum

Pipette 1 cc. of serum to a thick walled Pyrex test-tube (200×25 mm.). Add a small crystal of quartz, 1 cc. of 4 N H_2SO_4 , and 0.5 cc. of concentrated HNO_3 . Digest as one would in the usual micro-Kjeldahl method. When charring appears remove the flame and add carefully down the side of the tube a few drops of superoxol or concentrated HNO_3 . Digest again. If the solution does not clear, repeat the addition of H_2O_2 or HNO_3 and heating. After the solution has cleared, continue heating for a few minutes. Cool and add 4 to 5 drops of water, then pour quantitatively into approximately 15 cc. of freshly filtered reagent⁵ in a glass filter, weighed and stoppered as mentioned in the procedure for urine. Rinse the contents of the test-tube to filter with three 0.5 cc. portions of water, and finally two 3 cc. portions of reagent. Stir the solution in the filter till a precipitate appears and for several minutes thereafter. Withdraw the stirring rod, rinsing it with 3 cc. of reagent as it is withdrawn. Proceed with the determination as in the urine method. The calculation of results is the same as for urine.

The quantity of phosphate in 1 cc. of serum is so small that the increase in weight due to precipitation of uranyl phosphate introduces an error of less than 1 milli-equivalent of Na per liter.

Experimental Results

Table I gives some of the analytical results carried out in testing the accuracy of the method on a known salt solution.⁶ Analyses

⁵ The reagent should be shaken frequently preceding use to insure saturation.

⁶ The composition of the theoretical solution was per 100 cc.: NaCl , 9 m.-eq.; KH_2PO_4 , 21.6 m.-eq.; CaCl_2 , 1 m.-eq.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4 m.-eq.

1 to 6 show the accuracy of various dilutions. Analyses 7 to 10 were on a urine designated as Urine 1. Analysis of this sample by determining the combined Na and K as sulfates, the K as K_2PtCl_6 , and the Na by difference as Na_2SO_4 gave 3.08 milli-equivalents of Na per 100 cc. Analyses 7 and 8 were made with use of magnesia mixture for the removal of phosphate. Analyses 9 and 10 were made

TABLE I
Results on Urines of Usual Sodium Concentration

Analysis No	Solution analyzed	Size of sample	Precipitate minus blank	Na per 100 cc.	Theoretical m -eq. per 100 cc	Error
		cc.	gm.	m -eq.		per cent
1	Known salt solution	2	0.2803	9.12	9.00	+1.3
2	" " "	2	0.2800	9.10	9.00	+1.1
3	" " "	2	0.0920	2.99	3.00	-0.3
4	" " "	2	0.0920	2.99	3.00	-0.3
5	" " "	2	0.0271	0.881	0.90	-2.0
6	" " "	2	0.0270	0.881	0.90	-2.0
7*	Urine 1	5	0.2410	3.13	3.08*	+1.6
8*	" 1	5	0.2359	3.06	3.08*	-0.7
9	" 1	2	0.0941	3.06	3.08	-0.7
10	" 1	2	0.0937	3.04	3.08	-1.1
11	" 1 + NaCl	2	0.1930	6.28	6.24	+0.6
12	" 1 + "	2	0.1927	6.26	6.24	+0.3
13	" 1 + K_2HPO_4	3	0.1397	3.03	3.08	-1.6
14	" 1 + "	3	0.1402	3.04	3.08	-1.3
15*	" 1	2	0.0932	3.03	3.08	-1.6
16*	" 1	2	0.0943	3.05	3.08	-1.0
17	" 2	2	0.1322	4.30		
18	" 2	2	0.1349	4.38		
19*	" 2	4	0.2639	4.29		
20*	" 2	3	0.2001	4.34		

The blank ranged from 0.0015 to 0.0002 gm.

* For significance see text.

by the procedure given in this paper. Analyses 11 and 12 were on samples of Urine 1 to which known amounts of NaCl had been added. Analyses 13 and 14 were on samples of Urine 1 to which K_2HPO_4 was added in order to test the completeness of PO_4 removal when present in large amount. Analyses 15 and 16 were on Urine 1, $HgCl_2$ being used in the procedure, and show that this does not interfere with the determination. Analyses 17 and 18 were on

Urine 2, containing protein, with the use of HgCl_2 as precipitant. Analyses 19 and 20 were on Urine 2, the protein being removed by digestion with H_2SO_4 as for a micro-Kjeldahl analysis instead of by HgCl_2 precipitation.

TABLE II
Results on Known Solutions and Urines Low in Sodium

Analysis No.	Solution analyzed	Size of sample	Precipitate minus blank	Na per 100 cc.	Theoretical m.-eq. per 100 cc.	Error
		cc.	gm.	m.-eq.		per cent
1	Known salt Solution 1	5	0.1748	2.27	2.25	+0.9
2	" " " 1	5	0.1740	2.26	2.25	+0.4
3	Urine 3	10	0.6426	4.18	4.12*	
4	" 3	10	0.6417	4.17		
5	Known salt Solution 2	8	0.2479	2.01	2.00	+0.5
6	" " " 2	8	0.2439	1.98	2.00	-1.0
7	Urine 4	2	0.0000	0.00		
8†	" 4	40	0.0638	0.1037		
9†	" 4	40	0.0676	0.1098		
10	" 4 + NaCl	10	0.0902	0.5860	0.5923†	
11	" 4 + "	10	0.0900	0.5860	0.5923†	
12†	" 4 + "	40	0.3600	0.5850	0.5923†	
13†	" 4 + "	40	0.3675	0.5970	0.5923†	

* Determined by direct method on 2 cc. sample.

† Potassium removed.

‡ Added NaCl = 0.4855 m.-eq. per 100 cc. Hence $0.4855 + 0.1068 = 0.5923$.

TABLE III
Results on Serum from Normal Individual

Serum No.	Sample analyzed	Precipitate minus blank	Na per liter
		gm.	m.-eq.
1	1.0	0.2165	140.7
1	1.0	0.2158	140.4
1	1.0	0.2165	140.7
1	1.0	0.2163	140.6

Table II gives some of the results on solutions and urines low in sodium. Analyses 1 and 2 were on the known salt solution used in Table I but diluted so that 5 cc. were taken and concentrated

before addition to the reagent. Analyses 5 and 6 were on a known salt solution⁷ so high in potassium that it interfered and had to be removed, the results otherwise being about 9 per cent too high.

Table III shows the values obtained on a normal human serum.

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⁷ The composition of the theoretical solution was per liter: NaCl, 20 m.-eq.; CaCl₂, 10 m.-eq.; KH₂PO₄, 30 m.-eq.; MgSO₄·7H₂O, 40 m.-eq.; KCl, 131 m.-eq.

THE RELATION OF GLYCOGEN TO WATER STORAGE IN THE LIVER*

BY EDWARD M. BRIDGE AND E. M. BRIDGES

*(From the Department of Pediatrics, Johns Hopkins University, and the
Harriet Lane Home of the Johns Hopkins Hospital, Baltimore)*

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A relationship has been recognized for many years between water storage in the body and the metabolism of carbohydrate foods. As early as 1769 Stark (1) observed an extraordinary gain of 8 pounds in his own weight during the 5 days subsequent to changing his dietary from meat to flour. Numerous other investigators have confirmed and amplified this finding, so that at present the validity of the observation can hardly be questioned. That the phenomenon has an important bearing on the therapeutic effect of the high fat diets in controlling epileptic seizures has been shown recently (2). A clearer understanding of the underlying mechanism of such a reaction might conceivably aid in the management of high fat diets.

The fact that diets high in carbohydrates tend toward a retention of water in the body while high fat diets favor water elimination can be demonstrated by several clear cut experiments. Benedict and Milner (3) in 1907 reported a complete metabolism experiment on a man who performed a constant amount of work each day, and whose diet was suddenly changed from one in which carbohydrate furnished 66 per cent of the total calories to one which contained practically the same proportion of calories in fat. Accompanying the change there occurred a tremendous loss of water from the body with a corresponding weight loss.

The experiments of Gamble, Ross, and Tisdall (4) on the chemical changes resulting from a fast also illustrate the phenomenon. During the early days of fasting there occurred a very striking

* This work was made possible by a grant from the Epilepsy Medical Research Fund.

loss of body water, largely extracellular in origin. When the fast was broken by giving carbohydrate—in energy content far below the caloric needs—a gain in weight resulted accompanied by a retention of water. Hoelzel (5) observed the same phenomenon on himself as a result of eating carbohydrate foods with a generous amount of table salt at the end of a prolonged fasting period. Much water was retained as demonstrated by the intradermal salt solution test, and even by visible edema. Rubin (6), in this laboratory, has shown by chemical studies of retention that the abrupt gains in the weight of marantic infants after suddenly increasing the carbohydrate in the diet were largely due to water storage. Many other examples might be cited but these few are sufficiently striking to render almost certain the existence of some interrelationship in the metabolism of carbohydrate, fat, and water.

In explanation of this relationship the obscure statement is usually made that glycogen storage in the body is accompanied by a retention of water; and, conversely, when the glycogen stores in the body are depleted, water is eliminated. Every gm. of glycogen is said to bind 3 gm. of water. This explanation has seemed so adequate on superficial examination that the experimental evidence on which the conclusion was originally based has been completely overlooked. In view of the increasing application to medical therapeutics of the principles underlying these changes it seems justifiable to reinvestigate the evidence on which this conclusion was based.

The calculations which gave the ratio of 1 gm. of glycogen to 3 gm. of water, first published in 1906 by Zuntz, Loewy, Müller, and Caspari (7) in their voluminous studies entitled "*Höhenklima und Bergwanderungen*," were based not on their own experimental observations, but on previously published results of Pavy (8). The following average figures taken from Pavy, served as a basis for their calculations:

"1. 17 gm. liver with0.0 gm. glycogen
2. 33 " " " $33 \times 0.072 = 2.4$ " "
3. 64 " " " $64 \times 0.159 = 10.18$ " "

In the first instance a deposition of 2.4 grams of glycogen increased the liver weight by 16 grams, in the second case 10.18 grams of glycogen raised the weight of the liver by 47 grams. Every 100 grams increase in the weight

of the liver corresponds to a glycogen storage of 15 grams in the first case and 21.7 in the latter. . . . Some experiments of Pavy's using rabbits give an analogous result: an increase in weight of 100 grams corresponds to a storage of 28.2 grams of glycogen. . . . The consumption of one gram of glycogen lowers the body weight by four grams. . . . We may assume that with the storage of glycogen in the muscles the water content of the muscle is similar to that of the liver, so that here also approximately three parts of water are stored with one part of glycogen. . . ." (7)

If one considers this evidence in the light of modern physiology and chemistry the fallacies of the conclusions become obvious. In the first place the method of analysis for "amyloid substance" or glycogen was very crude indeed; 34 years later even Pavy himself recognized this and called attention to the fact with the statement that "glycogen [method] had not then attained the satisfactory position of that now at our disposal" (9). The finding of a glycogen content of the liver as high as 25 per cent can only be accounted for by the crudity of the method used, for many other substances were undoubtedly included in the precipitate which was weighed for glycogen. At that time, also, the rapidity with which glycogen leaves the liver after death was just beginning to be recognized. Most livers were examined immediately after death, but at least three of them not until some time later. Such wide variations occurred between different animals that the importance attached to the average values must be greatly minimized. As judged by our present chemical methods these results can only be interpreted as showing general tendencies; as absolute values they can mean nothing.

Secondly, since no determinations of the water content of the livers were made either by Pavy or Zuntz, but calculations based on the size of the liver in respect to body weight were used instead, it seems obvious that any conclusions drawn must be very inaccurate. Such calculations must be based on the assumption that the relative size of the liver in respect to body weight is constant; or, in other words, that the amount of liver tissue or liver protein is constant per unit of animal weight. An increase in the liver water or glycogen would be expected simply to cause the organ to swell, and would be associated with a decreased concentration of liver protein.

EXPERIMENTAL

In the experiments reported here rabbits have been subjected to such dietary measures as are known to affect the glycogen and water reserves of the body. At the end of the dietary period, usually several days, the animals were killed, the livers removed immediately, weighed, and samples taken for the analysis of glycogen, water, total nitrogen, and ether extractives. The weight of the intestinal contents was deducted from the total weight of

TABLE I
Analyses of Livers of Rabbits under Various Dietary Conditions

Rabbit No.	Weight*	Liver weight	Percentage of fresh liver				Undetermined substances	Diet
			H ₂ O	Protein	Glycogen	Fat		
	kg.	gm.						
74	2.3		72.4	13.2	10.6	1.9	1.9	Glucose and Casein
61	3.0	91	72.0	13.6	9.7	2.1	2.6	"
64	2.1	71	70.8	17.5	8.0	2.4	1.3	" and Casein
51		50	70.8	19.3	6.6	3.0	0.3	Normal
52	1.6	54	71.4	17.0	6.6	3.7	1.3	"
55	1.8	66	72.7	19.4	5.0	1.3	1.7	"
53	2.4	71					2.6	"
63	2.3	64	74.4	16.2	4.7	2.5	2.2	Glucose and insulin
12	2.5	84	70.5	18.7	2.2	5.2	3.4	40 per cent cream and Casein
11	3.3	98	75.0	17.5	0.4	2.5	4.6	40 " " " " albumin
54	1.7	54	73.0	20.3	1.1	3.6	2.0	Refused food
02	2.2	55	75.5	19.8	1.6	2.9	0.2	" glucose diet
01	2.3	54	70.5	20.0	0.4	5.2	3.9	Fasted
03	2.5	55	68.5	22.9	0.5	6.5	1.6	"

* Weight is recorded after removal of the intestinal contents.

the animal to make the latter represent, as nearly as possible, the active tissue of the body. No attempt was made to estimate the inactive fat tissue of the depots. Water was determined by drying samples of liver to constant weight, usually 4 to 7 days. Total nitrogen was determined by the macro-Kjeldahl method and the factor 6.25 used to calculate the protein. A micro adaptation of Pflüger's method was used for glycogen, the final determination of glucose being made with Folin's micro method (10). The ether-

soluble fraction was extracted in a Soxhlet apparatus, filtered, evaporated at low temperature, dried *in vacuo*, and weighed.

Results

In Table I are given the analytical data obtained from the livers of rabbits which had been fed on various diets; Table II shows these findings calculated on the basis of a kilo of animal weight. It will be seen from the results that the conclusions drawn by Zuntz and coworkers are not warranted. In the first place the basic assumption of a constant ratio of liver weight to body weight is not supported as such nor does it hold when considered on the basis of the amount of liver protein per kilo of animal weight. The latter figure varied from 7.0 to 4.1 gm. even in the small series of animals presented here. In the original figures of Pavy the size of the liver will also be seen to vary within wide limits, and to depend on the glycogen content only in a general way.

From the data in Table I it should be noted that, in general, a percentage change in one substance is offset by a change in the opposite direction in one or more of the other components. For example, when the glycogen content is highest, the protein concentration is lowest, and conversely, when the glycogen is low, the protein is usually high. The percentage of water and fat varies much less. The experimental error involved in such determinations is necessarily rather large, as illustrated by the values for the undetermined substances (salts, glucose, etc., and the additive errors). However, in general it seems obvious that, considered from the view-point of percentage composition of the liver, an increase in glycogen is offset by a reduction of the other constituents, and not only by a change in the percentage of water.

If instead of considering percentage composition of the liver, the total quantity of protein, glycogen, and water is calculated per kilo of body weight a somewhat different picture is produced. From the figures in Table II which are calculated in this way, it will be seen that the total liver water per kilo of animal weight is fairly constant. The lowest figures were found in the fasted animals and suggest the possibility of some dehydrating factor being present during a fast which does not occur with a fat diet. Under the conditions of the experiment the glycogen was made to vary within wide limits. The extremes of the ratio gm. H_2O :

gm. glycogen (7.4 — 225) illustrate the wide divergence between these two components of the liver; while the one is practically constant, the other varies from nearly a maximum figure of 2.9 gm. down to practically 0. Thus it would appear that the total amounts of water and glycogen in the liver per unit of body weight bear no relationship to each other.

Such an absence of relation could have been anticipated from the results of Benedict and Milner (3) which demonstrate conclusively that following the change to a fat diet the loss of body water occurred without an appreciable loss of carbohydrate ma-

TABLE II
Calculations Based on 1000 Gm. of Animal Weight

Rabbit No.	Liver weight	Total liver			Gm H ₂ O
		Protein	Glycogen	H ₂ O	Gm. glycogen
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
61	30	4.1	2.9	21.8	7.4
64	33	5.8	2.6	23.4	9.0
51	29	5.2	1.9	20.6	11.0
52	33	5.6	2.2	23.6	11.0
55	36	7.0	2.1	26.2	12.5
63	28	4.5	1.3	20.8	16.0
12	34	6.3	0.75	23.2	31.0
02	25	5.0	0.4	19.0	47.0
54	31	6.3	0.3	22.3	74.0
03	22	5.0	0.1	15.1	151.0
01	23	4.6	0.1	16.2	162.0
11	30	5.1	0.1	22.5	225.0

terials from the body. Studies by Slater (11) of the chemical and physical properties of glycogen have also failed to demonstrate any unusual hydration phenomena. Thus from both direct and indirect evidence it seems entirely unlikely that glycogen and water storage in the body are interdependent; certainly there is no simple mathematical relationship.

What then is the explanation of such sharply defined reactions resulting from sudden changes in the proportions of fat and carbohydrate in the diet? No positive answer can be given at present. The known facts concerning the phenomenon suggest that some phase of the fat metabolism may be responsible rather than car-

bohydrate and glycogen changes. Whether substances of the nature of cholesterol, lecithin, and fatty acids may be responsible, as has been suggested by Achard (12) in the case of certain edematous states of the body awaits further experimentation.

SUMMARY AND CONCLUSION

The glycogen content of the liver of rabbits has been made to vary by modifications of the diet. Alterations in the percentage of glycogen in the liver were found to be offset by changes in one or more of the other components, especially proteins. Per unit of animal weight the total glycogen and water of the liver bore no relationship to each other. The findings do not substantiate the frequently quoted statement that with every gm. of glycogen 3 gm. of water are also stored in the body. In explanation of the shifts in body water which result from changes in the proportion of fat and carbohydrate in the diet, it is suggested that the mechanism is more likely to be found in the metabolism of fats rather than of carbohydrates.

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AN IMPROVED TECHNIQUE FOR THE PRODUCTION OF NUTRITIONAL ANEMIA IN RATS*

BY C. A. ELVEHJEM AND A. R. KEMMERER

(From the Department of Agricultural Chemistry, University of Wisconsin, Madison)

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Rats have been used extensively for the study of nutritional anemia during the past few years. Most workers had had difficulty in producing severe anemia in rats during the first generation until it was demonstrated in 1928 in this laboratory (1) that distinct anemia was readily produced in young animals when the proper precautions were taken. The procedure which was developed at that time and which has been used continuously since then is essentially the following. Litters of rats from the stock colony are weaned between 3 and 4 weeks of age and placed in cages provided with screen bottoms and fed whole cow's milk *ad libitum*. The rats are continued on this diet until they are distinctly anemic, when they are segregated for individual feeding. When this method is used it generally takes 6 to 8 weeks to reduce the hemoglobin content of the blood to 3 to 4 gm. per 100 cc.

The recent work on iron metabolism has demonstrated that the milk anemia produced in rats by this procedure can be cured by the addition of iron and copper. Consequently, the production of anemia in rats fed a milk diet is due to a gradual diminution in the reserve supply of iron and copper present in the rat at the weaning time. The amount of iron and copper actually present in the rats when they are weaned, therefore, predetermines the length of time necessary for the production of severe anemia.

The work of Bunge (2) and Abderhalden (3) has led to the general conclusion that the iron content of a suckling animal decreases during the nursing period. This conclusion holds when

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the amount of iron is expressed on a percentage basis, but not when the total amount of iron present in the body is considered. Smythe and Miller (4) working with rats found the absolute amount of iron to increase from 0.279 mg. at birth to 0.806 mg. at 20 days of age.

Lindow, Peterson, and Steenbock (5) studying the copper metabolism of rats, found a similar increase in the absolute amount of copper during the suckling period. They found the average copper content per animal to be 0.0108 mg. at birth, and 0.0648 mg. when the animals were 26 to 27 days of age. Skinner, Peterson, and Steenbock (6) found an even greater increase in the manganese content of rats during similar periods. We were therefore interested to determine whether this increase in iron, copper, and manganese was due to a very efficient utilization of the small quantities of these elements in the mother's milk or to ingestion of some of the mother's rations during the suckling period. The latter suggestion seems to be the more plausible explanation since the increase in the amounts of these elements was greatest after the young were 12 days of age, that is, after the young had opened their eyes. It was through an attempt to answer this question that the following procedure for the production of nutritional anemia in rats was developed.

EXPERIMENTAL

Pregnant rats receiving the stock colony ration were removed shortly before parturition to individual cages equipped with screen bottoms. Screening two mesh to the inch was found to be satisfactory. A part of the screen was covered with a thin board upon which was placed a roll of cotton in which the young could be born. The rat was given no dry food in this cage but received whole cow's milk *ad libitum*. The mother was removed to a separate cage every day for feeding the dry ration and returned to the young after being thoroughly brushed to remove any adhering feed. In this procedure the young obtained only the mother's milk until they were 12 days old. After they opened their eyes they also consumed some of the cow's milk but they came in contact with no other food.

The young generally consumed definite amounts of the cow's milk when they reached the age of 17 days. The mother was

removed when the young were 21 days of age. The rats after weaning were continued on the whole milk and the hemoglobin content of their blood determined frequently. The blood was obtained by bleeding from the tail and the hemoglobin determined by the Newcomer method, a standardized Bausch and Lomb filter being used.

We were surprised to find that the young rats on this régime developed a severe anemia within 2 weeks after weaning. The weight and hemoglobin records of young animals handled according to this procedure are so constant that it hardly seems necessary to present individual records. The weight of the great majority of animals is between 30 and 35 gm. at weaning. There is a gradual increase to about 40 gm. after 1 week, and in some cases there is an increase during the 2nd week when the weight reaches 45 gm. More generally the weight begins to fall during the 2nd and 3rd weeks, and very few rats survive longer than 4 weeks. The hemoglobin content of the blood during the 2nd week on the milk diet is generally between 2.00 to 3.50 gm. per 100 cc. of blood. If no additions are made to the milk diet, the hemoglobin values continue to fall until death ensues. The hemoglobin content of a number of rats reached values below 2.00 gm., but the rats seldom survive more than a few days after the blood pigment has reached such low levels.

The individual records of rats taken from three different litters are presented in Table I. Litter 8 is included in the table because these rats developed anemia at a slower rate than any other group studied. Even these rats were distinctly anemic within 3 weeks after weaning. The anemia, therefore, develops at least 2 to 3 times as fast when this method is used as when the older technique is followed. Litter 24 is recorded because the rats in this group developed anemia as rapidly as any rats studied. The hemoglobin content of the blood reached values of 2.00 to 3.00 gm. per 100 cc. 8 days after weaning. The records of all other rats made anemic by this method fall between the figures tabulated for these two litters.

Litter 20 is included merely to show the effect of the size of the litter on the growth of the young. This litter contained nine rats and their weight at weaning was considerably less than that of smaller litters and the increment in weight after weaning was

very small. The hemoglobin content of the blood, however, decreased at about the same rate as that observed in litters of six. The practice of reducing the number of young in a litter to six when they are to be used for nutrition studies is generally accepted in all laboratories today but it is especially important in this case when the dry food consumed by the mother is rather limited.

TABLE I
Weight and Hemoglobin Records of Young Rats Made Anemic by Improved Technique

The readings are expressed in gm.

Litter No.	Days after weaning		Rat 33	Rat 34	Rat 35	Rat 36	Rat 37	Rat 38
8, six rats in litter	2	Body weight	34	31	37	32	35	32
	9		43	38	45	37	40	37
	16		43	33	47	33	43	36
	23		43	35	42	+	+	35
	13	Hb per 100 cc. blood	3.41	2.93	3.39	3.50	3.67	3.08
	23		1.75	2.08	1.91	+	+	1.75
24, five rats in litter	8	Body weight	Rat 71 40	Rat 72 34	Rat 73 40	Rat 74 39	Rat 75 34	
		Hb per 100 cc. blood	2.57	1.95	2.65	2.92	2.21	
	11	Body weight	Rat 50 20	Rat 51 25	Rat 52 20	Rat 53 22	Rat 54 22	Rat 55 25
		Hb per 100 cc. blood	2.15	2.07	2.24	1.91	2.22	2.15

Although the above results demonstrate that anemia can be produced much more rapidly when the new technique is used, further studies are necessary to show that the rats made anemic by this method are as suitable for studies on iron and copper metabolism as the rats prepared by the older method. A number of records have therefore been included to demonstrate the effect

of iron and copper supplements. Table II gives the results obtained when three rats from Litter 7 were fed as a group on milk which contained sufficient iron and copper to furnish each rat 0.5 mg. of Fe and 0.05 mg. of Cu daily. The rats were placed on the supplemented milk as soon as they were weaned. These rats grew quite well and the hemoglobin content of the blood soon reached normal values. The hemoglobin content of the blood was 6.00 to 8.00 gm. per 100 cc. when the rats were weaned; these values would have decreased to about 2.00 gm. if the rats had been continued on milk alone but when the iron and copper were added the figures increased to 13.00 to 15.00 gm. in a few weeks.

TABLE II

Weight and Hemoglobin Records of Young Rats Receiving Iron and Copper Supplements in Addition to Milk

The readings are expressed in gm.

Days after weaning	Body weight			Hb per 100 cc. blood		
	Rat 28	Rat 29	Rat 30	Rat 28	Rat 29	Rat 30
0	34	31	31	7.89	6.38	7.36
7	42	43	37	10.81	12.28	13.19
21	72	65	68	11.10	11.00	11.48
35	110	111	105	13.05	13.47	12.63
49	130	140	130	13.33	14.20	13.61
63	140	158	152	15.43	14.73	14.75

Table III gives the results obtained when rats were fed iron alone and iron plus copper after they had developed a severe anemia. The two animals that received the iron alone showed no improvement in the hemoglobin content of their blood over a period of 8 weeks and their weight remained practically constant during the same period. The rat receiving both iron and copper exhibited a normal blood stream 2 weeks after the additions were made to the milk, and grew at a much faster rate than the other rats. The results are similar to those obtained with rats made anemic by the older method.

These experiments show that the small increments in the iron and copper content of young rats during the suckling period, the presence of which tends to delay the development of anemia in the rats when continued on a milk diet after weaning, is due to the

ingestion of material other than the mother's milk and cow's milk. Much of this additional material undoubtedly comes from the dry feed of the mother's ration. The young obtain the feed during the early part of the nursing period from particles of food adhering to

TABLE III

Weights and Hemoglobin Records of Young Rats Receiving Iron and Copper Supplements after Developing Anemia

The readings are expressed in gm.

Days after weaning*	Body weight			Hb per 100 cc. blood		
	Rat 90	Rat 91	Rat 92	Rat 90	Rat 91	Rat 92
18	60	58	60	2.54	2.61	3.08
25	60	60	65	3.61	2.70	8.57
32	64	65	77	3.94	1.95	13.33
39	65	52	82	3.08	2.71	10.90
46	67	58	92	4.36	4.10	11.57
53	64	67	94	2.89	3.57	11.71
60	60	67	98	2.84	4.10	13.05
67	65	70	107	2.96	4.15	11.48
74	73	68	120	3.47	3.32	12.40
81	85	68	135	3.67	2.22	11.48

* On the 19th day after weaning Rats 90 and 91 received 0.5 mg. of Fe, Rat 92 received 0.5 mg. of Fe plus 0.05 mg. of Cu daily.

TABLE IV

Manganese Content of Rats Reared According to Old and New Techniques

Litter No	No. of rats in litter	Age	Average weight	Average Mn content	Technique
		days	gm.	mg.	
14	9	12	17.0	0.0130	Old
6	4	13	17.0	0.0026	New
11	9	12	13.0	0.0039	"
13	4	12	16.0	0.0034	"
16	3	21	51.0	0.0150	Old
25	2	21	28.0	0.0053	New
27	3	21	34.0	0.0064	"

the mother's fur. When they are old enough to move about the cage they may consume a certain amount of the refuse scattered about the cage or some of the dry ration directly from the feed containers.

The results presented in Table IV show that the improved technique which prevents the consumption of food other than milk is not only effective for the reduction of iron and copper stores in the young rats but that it is equally as valuable for decreasing the amount of manganese in young rats. We (7) have recently called attention to the importance of preventing manganese storage in young mice used for manganese metabolism studies. The manganese content of the rats was determined according to the methods used by Skinner, Peterson, and Steenbock (6). It is readily seen that the manganese content of the young rats, both when 12 and 21 days of age, is distinctly less when the new technique is used. The average manganese content of these rats is about one-half in the case of the 12 day old animals and somewhat less than one-half for the 21 day old rats of the average figures obtained by Skinner, Peterson, and Steenbock for rats of the same ages reared on shavings and in contact with the mother's ration.

SUMMARY

An improved technique for the rapid production of nutritional anemia in rats is outlined.

Figures are presented to show that rats made anemic by this method are suitable for iron and copper metabolism studies.

This technique may also be used for the production of young rats to be used for manganese metabolism studies.

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INEFFECTIVENESS OF PURIFIED GLUTAMIC ACID AS A SUPPLEMENT TO IRON IN THE CORRECTION OF NUTRITIONAL ANEMIA*

BY C. A. ELVEHJEM, H. STEENBOCK, AND E. B. HART

WITH THE COOPERATION OF EVELYN VAN DONK†

(From the Department of Agricultural Chemistry, University of Wisconsin, Madison)

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In a recent paper Drabkin and Miller (1) concluded that certain amino acids, especially arginine and glutamic acid, when added to a milk diet supplemented with iron are very effective for the relief of anemia. We naturally agree with their suggestion that certain organic groups, such as may be derived from amino acids, are necessary for the formation of hemoglobin. However, we question their conclusion that certain pure amino acids are active agents in the cure of anemia produced by a milk diet.

The distinct specificity of copper as a supplement to iron in the cure of nutritional anemia makes it difficult for one to explain the decided activity of amino acids such as arginine and glutamic acid, and the inactivity of amino acids such as alanine and histidine. It also seems improbable that glutamic acid can be a limiting factor in a milk diet when casein, an important constituent of milk, contains at least 20 per cent of this compound. The fact that several investigators (2-5) have raised hundreds of animals with normal hemoglobin levels on a diet of whole cow's milk supplemented with iron and copper demonstrates without doubt that milk is deficient only in iron and copper as far as hemoglobin formation is concerned. Therefore the activity of relatively large amounts of glutamic acid when added to a milk and iron diet, which is complete for hemoglobin building when traces of copper

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† E. R. Squibb and Sons Fellow.

are supplied, must be explained by the fact that glutamic acid has the same effect as copper. Before it is possible to conclude that large amounts of any organic compound can function in the same physiological capacity as copper, the *purity* of the compound in question must be investigated thoroughly. The compounds found to be active in hemoglobin regeneration may contain traces of copper while the inactive compounds must be exceedingly low in copper.

We (6) have demonstrated recently that the activity of the synthetic diets used by Drabkin and Waggoner (7) in anemia studies was due to the presence of copper in sufficient quantities to promote hemoglobin regeneration. It is a well known fact that many amino acids form definite and stable compounds with copper, thus the last traces of copper are probably removed from certain amino acids with some difficulty. Since very small quantities of copper promote hemoglobin regeneration in rats it is very essential that great care be taken to prevent the copper contamination during all feeding trials. In view of these facts we believed it necessary to repeat Drabkin and Miller's work under carefully controlled conditions.

EXPERIMENTAL

We limited our studies to only one of the two amino acids reported to possess the greatest activity, namely glutamic acid. Both glutamic acid hydrochloride and the free acid were used in the feeding trials. The glutamic acid used in this work was prepared in the usual manner but special precautions were taken in the recrystallization and purification of the original sample. The crude preparation was dissolved in water, decolorized with charcoal, and precipitated as the hydrochloride by saturation with washed HCl gas. The charcoal was extracted with HCl and washed with water before it was used in order to minimize copper contamination through this source. The glutamic acid hydrochloride crystals were filtered off on Jena glass filter crucibles and washed with cold concentrated HCl. The hydrochloride was dried in a vacuum desiccator over potash and sulfuric acid. The melting point of this preparation was 193–194° uncorrected.

It is impossible to say that this preparation was completely copper-free, but a 10 gm. sample analyzed according to the method

of Elvehjem and Lindow (8) contained an amount of copper insufficient to determine (less than 0.002 mg.). 100 mg. of the preparation were fed daily to rats.

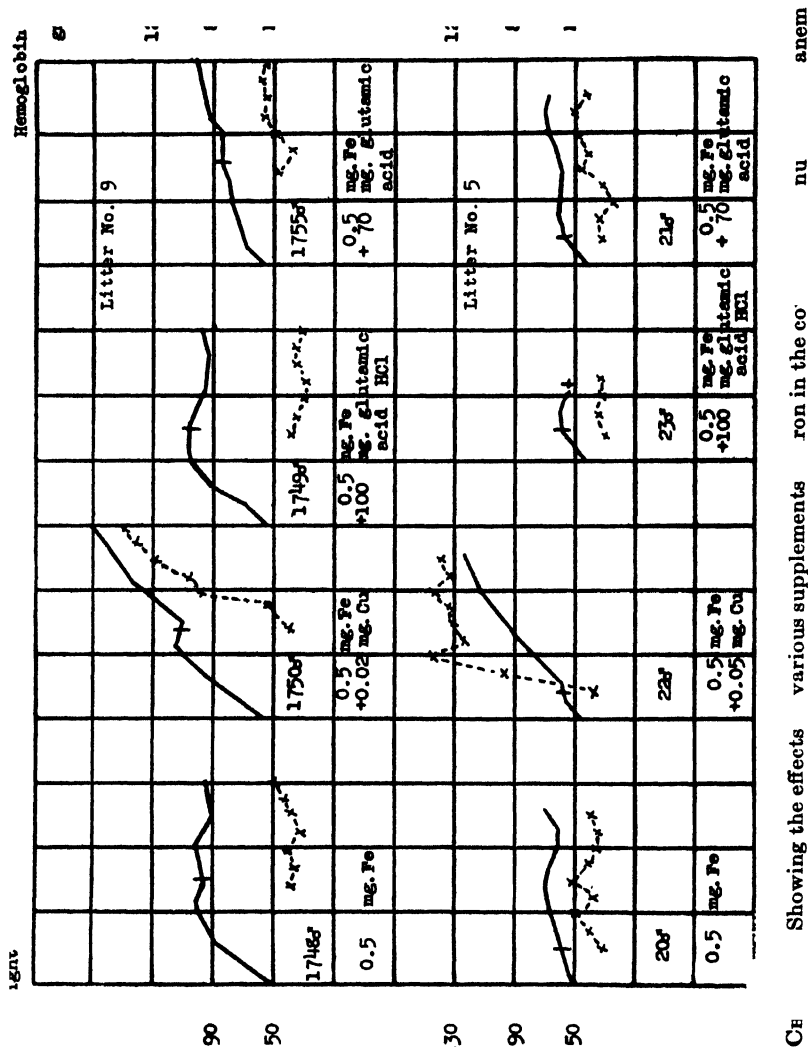
The free glutamic acid was prepared by suspending 10 gm. portions of the hydrochloride in a small quantity of water and adding the theoretical amount of copper-free NH_4OH necessary to remove the HCl . The precipitate formed was filtered off and washed thoroughly with ice-cold water. The melting point of this preparation was 198° uncorrected. This material was fed in 70 mg. quantities to rats daily.

These purified preparations were not analyzed for iron because they were fed to the experimental animals together with optimum amounts of this element. The presence of small additional amounts of iron would therefore have no further effect on hemoglobin regeneration.

Albino rats were used for all the experiments, but two different procedures were used for development of the anemia. The *first* group of animals was made anemic by the procedure which has been used in this laboratory (9) for several years. In this method the young rats are not placed on screens until they are weaned and consequently the anemia does not develop until they have been on a milk diet from 6 to 9 weeks. The *second* group of animals was made anemic by the modified method described by Elvehjem and Kemmerer (10) which produces a severe anemia within 2 weeks after weaning. The hemoglobin values were determined by the Newcomer method, a standardized Bausch and Lomb filter being used.

When the rats were distinctly anemic they were segregated in individual cages and each rat was given milk plus 0.5 mg. of Fe as FeCl_3 daily. The iron used was purified in the usual manner. In each litter one of the six rats was continued on iron alone, one was given copper in addition to iron, two were fed 100 mg. of glutamic acid hydrochloride plus iron, and two were given 70 mg. of glutamic acid plus the iron each day. Several litters of rats were used in the study but the records of only two litters are presented in Chart I. Litter 9 was taken from the *first* group of rats and Litter 5 from the *second* group of animals.

The results recorded in Chart I which are typical of all those obtained need no explanation. The responses made by the



animals that received the purified iron alone or the iron plus copper are identical with those previously recorded for animals receiving similar supplements. The records of the animals given glutamic acid plus the iron demonstrate that this amino acid is not an active supplement to iron for the cure of nutritional anemia.

It is impossible for us to explain the positive results obtained by Drabkin and Miller when glutamic acid was fed. There are, however, certain facts that are rather obvious in their paper, which may aid in the explanation of their results. A 0.5 gm. sample was used for the copper analysis of most of the amino acids studied. Since it is difficult to detect less than 0.005 mg. of copper unless special precautions are taken, the amount of sample used by them would seem too small for an accurate assay of their preparation. It is also impossible to determine from their charts whether negative controls were maintained for each litter. This is a very important factor since different litters may vary in their reserve supply of copper.

SUMMARY

Relatively large amounts of pure glutamic acid, added as a supplement to whole milk and iron, did not correct nutritional anemia in the rat.

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THE PREPARATION OF STANDARD ACID HEMATIN SOLUTIONS FROM HEMIN*

BY C. A. ELVEHJEM

(From the Department of Agricultural Chemistry, University of Wisconsin, Madison)

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The need of a method which is simple, rapid, and accurate for the determination of blood hemoglobin is evident to all who have had occasion to make a large number of such determinations. The general use of the Newcomer method today in clinics and laboratories demonstrates that it has met to a large extent the requirements of such a method. The utilization of a piece of brown-colored glass of definite thickness as a permanent standard eliminates the necessity of preparing standard hematin solutions, a procedure entailing a considerable amount of work, especially when the solutions are made from blood.

Although the merits of the Newcomer method are recognized by most workers, there are certain disadvantages which must be considered. Robscheit (1), in a survey of the methods used for hemoglobin determinations, states, "Newcomer's method obviates many difficulties heretofore observed with other procedures and gives good results with the glass 0.96 mm. in thickness, although the color is quite pale." She found that the use of a heavier glass gave figures which were not so satisfactory. In view of these facts, the workers in her laboratory prefer to use liquid acid hematin standards. Osgood and Haskins (2) reached similar conclusions.

We have used the Newcomer method almost exclusively in our laboratory, although we have been aware of the disadvantages mentioned. We have also found that the filters placed on the market the past few years vary to a large extent and must be standardized before they can be used for accurate determinations.

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The Bausch and Lomb Optical Company (personal communication) recognizes the importance of calibrating all of the filters which they have furnished since they have sold four different lots of filters, each lot having been calibrated in a different way and at a different time. We have in our laboratory discs which vary as much as 12 per cent in the ultimate measurement of hemoglobin per 100 cc. of whole blood. In order to obviate any uncertainty as to the accuracy of the discs used in the different laboratories, a method for the rapid determination of the hemoglobin equivalent of these filters is needed. It is the purpose of this paper to outline a simplified method for the preparation of standard acid hematin solutions which may be used for the estimation of hemoglobin directly or for the standardization of any hemoglobinometer based on the acid hematin principle.

The acid hematin method, which entails the conversion of the hemoglobin in the blood sample to acid hematin with 0.1 *N* HCl and the comparison of the color produced with standard tubes of acid hematin, came into general use due to the work of Sahli (3). Cohen and Smith (4), who modified the original method by introducing the use of the colorimeter for comparing the intensity of the colors, also demonstrated that the acid hematin prepared from the blood of one species may be used for the determination of hemoglobin in the blood of a number of other species. Most of the standard acid hematin solutions used in the past have been prepared from blood obtained from any available animal, and the hemoglobin equivalent of each solution has been calculated from the oxygen capacity of the blood which is determined by the Van Slyke method. The calibration of the disc used in the Newcomer method (5) is also based on the oxygen capacity of blood. The standard solution, devised by Osgood and Haskins (2), which contains only inorganic material, is standardized in a similar manner.

Workers who have improved the acid hematin method have stressed the technique of making the estimations rather than the chemical changes involved in the formation of acid hematin. The term hematin has been used for over a century and it has generally been employed to designate the pigment part of the hemoglobin molecule. Anson and Mirsky (6) have suggested that the so called acid hematin formed when blood is treated with an acid cannot be the free iron compound; *e.g.*, the hematin group

unattached to other compounds. The evidence for their conclusion is based upon the fact that a soluble compound is formed when blood is treated with 0.1 N HCl and an insoluble compound is formed when a solution of hemin in NaOH is treated with acid of the same concentration. They call the free iron compound heme and believe the soluble compound (acid hematin) to be oxidized heme united with a nitrogen compound.

Keilin (7), however, has shown that if 0.2 per cent gum arabic is added to a solution of hemin in NaOH before acidification with HCl, the iron compound does not precipitate but remains in colloidal suspension protected from precipitation by the gum arabic. In his preparation the fluid remained clear and transparent, and gave the same absorption spectrum as acid hematin prepared from hemoglobin. It is, therefore, evident that the acid hematin produced from blood is a colloidal suspension of insoluble hematin protected from precipitation by the globin and that the introduction of a new term to distinguish a different iron compound is unnecessary.

It should therefore be possible to prepare a standard acid hematin solution from pure hemin, provided a protective colloid is used, and thereby eliminate the necessity of always preparing hematin solutions from blood. This procedure allows the hemoglobin equivalent to be calculated directly from the iron content of the hemin. When the iron content of the hemin preparation is once determined a large number of solutions may be prepared from the same sample, while when blood is used the oxygen capacity must be determined each time.

EXPERIMENTAL

The hemin used in this work was prepared by the following method: 2 liters of glacial acetic acid were placed in a 5 liter flask and 100 cc. of water and 0.25 gm. of NaCl were added. The flask was heated in a boiling water bath until the temperature of the acetic acid reached 90°. The flask was then removed from the bath and 850 cc. of defibrinated horse blood were added slowly from a dropping funnel. The flask was shaken continuously during the addition of the blood. The mixture was heated to 90° and kept at that temperature for 15 minutes after which the hemin crystals were allowed to settle overnight. The supernatant liquid

was syphoned off and the crystals resuspended in dilute acetic acid. After allowing the crystals to settle overnight the clear liquid was syphoned off. This washing process was repeated with water, dilute HCl, and finally water. The washed crystals were filtered off and dried at room temperature.

The iron content of this preparation was found to be 7.4 per cent. The theoretical iron content calculated from the accepted empirical formula for hemin is 8.6 per cent. The lower iron content is largely due to the presence of a certain amount of moisture since the crystals were dried at room temperature, but the preparation may also contain a slight amount of other impurities since it was not recrystallized.

30 mg. of hemin were dissolved in sufficient dilute NH_4OH to bring about complete solution and the solution was diluted to 50 cc. A 10 cc. aliquot equivalent to 6 mg. of hemin was measured out, 5 cc. of a 5 per cent solution of gelatin were added, and the solution was diluted to 250 cc. with 0.1 N HCl. The solution of acid hematin prepared in this manner appeared to be very similar to that produced from blood. Since 1 cc. of blood contains approximately 6 mg. of hemin, the color intensity of the hematin solution was approximately the same as that obtained in the ordinary hemoglobin determinations when a dilution of 1 to 250 is made. The acid hematin solution was allowed to stand 24 hours and then the hemoglobin equivalent was determined by the Newcomer method.

Another 10 cc. aliquot was evaporated to dryness, ashed, and analyzed for iron. Since hemoglobin contains 0.335 per cent iron, the hemoglobin equivalent was calculated directly from the amount of iron found present. A large number of such determinations have been made but only a few typical results are given in Table I. Since the iron determinations were made on aliquots of each hemin solution no attempt was made to weigh exactly the 30 mg. of hemin used. The iron content of different solutions therefore varies slightly, but the duplicate determination on the same solution agrees very well.

The hemoglobin equivalent was determined with two different discs, and both results are recorded in Table I. The two filters were purchased from the Bausch and Lomb Optical Company. Filter 1 was purchased the early part of this year and Filter 2 was

purchased 4 years ago. It is readily seen from these results that the two discs vary in their hemoglobin equivalent to a considerable extent. Other discs in our laboratory show similar variations but not to such a great extent. Results also show that the hemoglobin equivalent for the various hematin solutions determined by the use of Filter 1 check very well with those calculated from the iron content. Filter 1 is the disc marketed by the Bausch and Lomb Optical Company at the present time. They have used a very elaborate procedure in determining the factor for this filter and, therefore, hope it is the most accurate of all the discs that have been placed on the market (personal communication).

TABLE I
Hemoglobin Equivalents of Acid Hematin Solutions

In each case a 10 cc. aliquot taken from solutions containing 30 mg. of hemin per 50 cc. was used for the iron determination and for the preparation of the acid hematin solution.

Solution No.	Amount of hemin	Iron content	Hb calculated from iron	Newcomer method	
				Filter 1	Filter 2
	<i>mg</i>	<i>mg.</i>	<i>gm</i>	<i>gm.</i>	<i>gm</i>
1	6	0.430	12.8	12.8	10.9
	6	0.433	12.9	12.8	10.9
2	6	0.447	13.3	13.1	11.2
	6	0.445	13.2	13.1	11.4

If the hemoglobin equivalent of a disc does not check with that of a standard acid hematin solution, the disc need not be discarded, but a factor may be introduced to convert the reading obtained to the correct hemoglobin value. For example, a factor of 1.17 will convert the results obtained with Filter 2 to values which agree with Filter 1 and the standard hematin solutions.

These results demonstrate that pure hemin may be used for the preparation of standard acid hematin solutions and that the hemoglobin equivalent of these solutions may be calculated from the iron content of the hemin used. In order further to substantiate this conclusion the hemoglobin content of several different samples of blood was determined by the following methods: (1) Newcomer, Bausch and Lomb Filter 1 with conversion table (H 236, 1×30)

being used, (2) standard acid hematin solution, and (3) oxygen capacity. About 10 cc. of blood were drawn from the animal and oxalated so that the same sample could be used for all methods. In the first and second methods the blood was diluted 1:250 with 0.1 N HCl and allowed to stand 40 minutes before making the colorimetric readings. The acid hematin solution used in the second method was the one designated as Solution 1 in Table I, and the value of 12.85 gm. of hemoglobin was taken as the hemoglobin equivalent.

The oxygen capacity of the various samples was determined by the use of Barcroft differential manometers. The manometers were properly calibrated and the flasks were thoroughly cleaned before being used. 2 cc. of ammonia solution (4 parts ammonium hydroxide, sp. gr. 0.88, in 1000 cc. of distilled water) were placed in each flask and 1 cc. of the blood sample was added. The contents of each flask were well shaken so as to take the blood and saturate it with oxygen. In the right-hand flask a small vial (as used by Keilin (8)) containing 0.2 cc. of saturated ferricyanide was suspended from the absorption tube. Both flasks were attached to the manometer and the apparatus placed in the water bath for 5 minutes. The taps were then closed, the manometer was read, and the ferricyanide upset into the flask. The apparatus was shaken in a horizontal direction, and the reading was taken when the constant maximal pressure was reached. Duplicate determinations at 20° and 37° were made for each sample of blood. The oxygen capacity of the blood was calculated by using the constant for each apparatus and by correcting for temperature and pressure. The hemoglobin content of the blood was obtained by dividing the oxygen capacity by 1.34.

The duplicate results obtained by the use of the three different methods on two samples of blood are given in Table II. These results are typical of a number of analyses. It is readily seen from this table that the values obtained by the three methods check very well. The figures obtained by the oxygen capacity method are slightly lower than those obtained by the other methods. However, the small discrepancy is probably due to the fact that Barcroft's differential method may give slightly lower results for the oxygen capacity of blood than the Van Slyke method (9). Acid hematin solutions prepared from hemin can therefore

be used as standards for hemoglobin determinations as well as for the standardization of the glass discs. Liquid standards have the advantage that the intensity of the color may be varied by changing the depth of solution in the colorimeter. The permanency of the liquid standards is naturally an important factor if they are to be used in hemoglobin determinations. Several solutions have

TABLE II
Hemoglobin Content of Various Samples of Blood Determined by Different Methods

Sample No	Gm. Hb per 100 cc. blood		
	Newcomer, Filter 1	Standard acid hematin	Oxygen capacity
1 a	10.7	10.7	10.5
1 b	10.5	10.8	10.5
2 a	12.6	12.6	12.3
2 b	12.6	12.5	12.4

TABLE III
Effect of Age on Hemoglobin Equivalents of Acid Hematin Solutions

Date	Gm. of Hb	
	Solution 1	Solution 2
Mar. 28	Prepared	Prepared
" 29	12.62	12.63
" 30	12.91	12.77
Apr. 2	12.41	12.51
" 1	12.62	12.63
" 16	12.41	12.40
May 2	12.41	12.51
" 22	12.41	12.40

been kept in the laboratory for 2 months with no definite change in the intensity of their color. Table III gives the hemoglobin equivalents for two of the solutions determined at various intervals.

There is no reason for displacing the Newcomer method with procedures entailing the use of liquid standards providing the glass discs used in the Newcomer method are properly calibrated. The method outlined in this paper is so simple that the calibra-

tion of the Newcomer discs in use becomes an easy procedure. The comparison of hemoglobin values obtained in different laboratories is only possible when all workers use a similar method and the standards used are properly calibrated.

SUMMARY

A method for the preparation of standard acid hematin solutions from hemin of known iron content is outlined.

The hematin solutions may be used as a standard for the determination of hemoglobin or may be used to calibrate the glass discs used in the Newcomer method.

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THE DETERMINATION OF CALCIUM BY ALKALIMETRIC TITRATION

II. THE PRECIPITATION OF CALCIUM IN THE PRESENCE OF MAGNESIUM, PHOSPHATE, AND SULFATE, WITH APPLICATIONS TO THE ANALYSIS OF URINE

BY CYRUS H. FISKE AND MILAN A. LOGAN*

(From the Biochemical Laboratory, Harvard Medical School, Boston)

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The investigation of McCrudden¹ in 1909 is the starting point of practically all methods which have in the meantime been devised for the determination of calcium in urine, or in other biological products where the chief difficulty lies in the separation of this element from both phosphate and magnesium. Such products—and particularly urine—may however contain, in proportion to the calcium, much larger quantities of these interfering substances than were present in any of the known mixtures tested by McCrudden. In addition to the sources of error which have so far been generally recognized, there is in the case of urine of low calcium content a further complication in the fact that an excess of sulfate or phosphate tends to increase the solubility of calcium oxalate, or at least to delay precipitation.

As far as the analysis of urine is concerned, it is a matter of some consequence to note that the technique recommended by McCrudden for this special purpose² differs from his original procedure¹ in some respects, especially in the concentration of oxalate present during the precipitation. In the urine method the oxalate concentration used is less than 0.02 N, an amount which has been found too small to bring about complete precipitation of the calcium from mixtures of high magnesium content, owing to the effect of

* Fellow of the Forsyth Dental Infirmary.

¹ McCrudden, F. H., *J. Biol. Chem.*, **7**, 83, 201 (1909-10).

² McCrudden, F. H., *J. Biol. Chem.*, **10**, 187 (1911-12).

magnesium salts on the solubility of calcium oxalate.³ Since the prevention of contamination by magnesium ammonium phosphate is another function of the precipitating agent, it is clear that either high or low results, depending upon the composition of the solution, may be expected if the calcium is precipitated with only a slight excess of oxalate.

The difficulties associated with the separation of a small amount of calcium from a much larger amount of magnesium cannot be remedied by merely increasing the concentration of oxalate to the point where the precipitation of calcium is complete, for magnesium oxalate will then come down as well.^{3,4} That this undesirable result may be forestalled by the use of a large excess of the precipitant, leading to the formation of soluble complex magnesium ammonium oxalates, has long been known in principle,⁵ but the device has not been used to good effect until quite recently, and so far has been applied only to the relatively simple problem of separating calcium from magnesium alone. In the method of Bobtelsky and Malkowa-Janowskaja,⁶ apparently the best one yet suggested for the technical analysis of magnesite and related products, a tremendous excess of ammonium oxalate is prescribed (15 to 25 gm. of the solid salt per 100 cc. of solution). For micro-analytical work this arrangement is not suitable, in the first place because the precipitation and filtration must be conducted at or near the boiling point, and also owing to the fact that magnesium cannot be determined in the filtrate in the presence of so large a quantity of oxalate, which consequently must first be decomposed.⁷ We have accordingly endeavored to determine whether a pure calcium oxalate precipitate can be obtained at room temperature in quantitative yield, in the presence of large amounts of phosphate and magnesium (and also sulfate), using an amount of

³ Blasdale, W. C., *J. Am. Chem. Soc.*, **31**, 917 (1909). Fischer, W. M., *Z. anorg. u. allg. Chem.*, **153**, 63 (1926).

⁴ Rodt, V., and Kindscher, E., *Chem. Ztg.*, **48**, 953 (1924). Luff, G., *Z. anal. Chem.*, **65**, 439 (1924-25).

⁵ Souchay, A., and Lenssen, E., *Ann. Chem.*, **100**, 308 (1856). Wittstein, G. C., *Z. anal. Chem.*, **2**, 318 (1863).

⁶ Bobtelsky, M., and Malkowa-Janowskaja, *Z. angew. Chem.*, **40**, 1434 (1927).

⁷ See Hall, W. T., *J. Am. Chem. Soc.*, **50**, 2704 (1928).

oxalate small enough to permit complete precipitation of magnesium ammonium phosphate from the filtrate if desired.

The urine of fasting animals may contain, per mg. of calcium, as much as 12 mg. of magnesium, 150 mg. of phosphorus, and 150 mg. of sulfur.⁸ As a result of numerous experiments on pure salt mixtures of corresponding composition⁹ it has been found that 0.16 N oxalate, the equivalent of 1 per cent oxalic acid, will suffice (at pH 5) to precipitate the calcium completely and to prevent contamination by magnesium, provided that the following conditions are fulfilled: (1) that the concentration of calcium in the solution is not less than 0.01 or more than 0.03 mg. per cc., and (2) that the solubility of magnesium oxalate is further increased by the addition of ammonium chloride (or nitrate), which has practically its maximum effect at a concentration of 0.5 N.

The procedure which we have adopted for the precipitation of calcium oxalate and for the subsequent steps of the analysis, concluding with the titration of the oxide,¹⁰ will now be described. In many cases ashing is required before the calcium is precipitated, but as the preliminary treatment varies with the nature of the material to be analyzed this aspect of the subject will be discussed later under separate headings.

Reagents

Ammonium Chloride, 5 N—268 gm. are dissolved in water and made up to 1 liter.

Oxalic Acid, 2.5 Per Cent—25 gm. of crystalline oxalic acid are dissolved in water and made up to 1 liter.

Ammonium Oxalate, 3 Per Cent—30 gm. are dissolved in water and made up to 1 liter. The solution should be filtered the day after it has been prepared.

⁸ In special cases (e.g., in the urine of animals fed large quantities of meat) the conditions may be even more extreme. The determination of calcium in such material can be carried out more rapidly by the use of a double precipitation method (p. 224).

⁹ Hecht has reported good results with mixtures containing calcium, magnesium, and phosphate in similar proportions, but his work unfortunately cannot be duplicated inasmuch as explicit directions are not given (Hecht, G., *Biochem. Z.*, **143**, 342 (1923)).

¹⁰ Fiske, C. H., and Adams, E. T., *J. Am. Chem. Soc.*, **53**, 2498 (1931).

Sulfuric Acid, Approximately 10 N—285 cc. of concentrated sulfuric acid (sp. gr. 1.84) are added to 800 cc. of water.

Concentrated Nitric Acid (Sp. Gr. 1.42).

Nitric Acid, Approximately 1 N—63 cc. of concentrated nitric acid are added to 960 cc. of water.

Ammonium Hydroxide, 5 N—This is prepared by adding concentrated ammonium hydroxide from a dropping funnel to stick sodium hydroxide, and collecting the gas in water (in a cooled receiver). The solution thus prepared is diluted to the desired concentration and kept in a paraffined bottle.

Hydrogen Peroxide, 30 Per Cent—Merck's superoxol.

Alcohol, 95 Per Cent.

Methyl Red—Saturated alcoholic solution.

0.02 N Hydrochloric Acid.

0.02 N Sodium Hydroxide—A paraffined bottle¹¹ of 1 liter capacity is provided with a 2-hole rubber stopper bearing (a) a soda-lime tube, and (b) a paraffined glass tube long enough to reach nearly to the bottom of the bottle. The upper end of the glass tube, which projects several cm. beyond the stopper, is drawn out to a bore of about 1 mm., and is closed with a piece of rubber tubing and a short glass rod. The bottle is nearly filled with distilled water, to which is added a sufficient amount of freshly prepared saturated sodium hydroxide solution to make a liter of approximately 0.02 N alkali. The rubber stopper with its attachments is *at once* inserted and sealed in with paraffin, and the contents of the bottle are thoroughly mixed by shaking. The standard alkali is withdrawn as needed by connecting the tip of a micro burette with rubber tubing, and applying suction¹² or pressure as preferred.

The precautions given above for the exclusion of carbon dioxide from the standard alkali may fail of their purpose unless the same degree of care is exercised in keeping the solution free of silicate, which like carbonate detracts from the sharpness of the end-point. For this reason the saturated solution of sodium hydroxide from which the standard is to be prepared should be made by dissolving alkali (of the purest quality obtainable) in water in a metal dish (preferably platinum), since an appreciable amount of

¹¹ Paraffin of fairly high melting point (about 55°) should be used.

¹² Folin, O., and Peck, E. C., *J. Biol. Chem.*, **38**, 287 (1919).

silicate may be introduced by even brief contact with glass while the solution is still hot. The thoroughly cooled solution may be transferred to a cylinder and let stand until the sediment has settled out, but this takes time and is not free from risk. A quicker, and safer, method is to filter off the sediment with suction. For this purpose a Jena glass filter is more convenient than a Buchner funnel, but the first portion of the filtrate may contain some silicate derived from the glass filter, and should be discarded. The remainder of the filtrate is collected in a test-tube, and is used immediately for the preparation of the standard.

By this procedure we have had no difficulty in preparing even 0.01 N alkali which will give substantially the same titration value (within 0.2 per cent) whether the indicator used is methyl red or phenolphthalein. Standards made from saturated alkali which has been prepared in glass receptacles in our experience will not meet this test.

Testing and Purification of Reagents

All the above reagents, with the exception of the standard alkali and acid, must be calcium-free. It is important that their purity in this respect be established in each case by a definitely specified procedure known to be delicate enough to reveal a significant amount of calcium. In addition, those reagents which are used for washing the calcium oxalate precipitate and for converting it to calcium oxide must be substantially free from impurities of any sort that will leave an alkaline residue on ignition.

The details of the various tests required are given in Table I, together with the method of purification to be used when the commercial article proves unsatisfactory. As far as our own experience goes, it is useless to spend time testing commercial ammonium hydroxide, which evidently takes up calcium from the soft glass bottles in which it is marketed, and to some extent even from hard glass. Dilute (5 N) ammonium hydroxide soon becomes quite worthless as a reagent for the determination of calcium if kept in an unparaffined receptacle.

Method

Precipitation—The directions following apply to the precipitation of 0.25 to 0.75 mg. of calcium (at a final volume of 25 cc.)

TABLE I
Method of Testing and Purifying Reagent.

Reagent	Test for Ca	Test for base	Method of purification
Alcohol.....	Evaporate 100 cc. to dryness; test residue*	Ignite 1 gm. solid salt in platinum. Dissolve residue with 1 cc. 0.02 N HCl, and titrate with 0.02 N NaOH to turning point of methyl red. At least 0.9 cc. 0.02 N NaOH should be required	Distillation
Ammonium chloride.....	Ignite 5 gm. solid salt in platinum; test residue*		Recrystallization from water See p. 214
“ hydroxide.....	Evaporate 50 cc. to dryness in platinum; test residue*		Recrystallization from water
“ oxalate	No test necessary		
Nitric acid.....	Evaporate 50 cc. concentrated acid to dryness; test residue*	Evaporate 10 cc. concentrated acid to dryness in platinum. Add 0.5 cc. 2.5 per cent oxalic acid, evaporate to dryness, ignite, and proceed as in case of ammonium oxalate	Distillation
Oxalic acid.....	Ignite 5 gm. crystals in platinum; test residue*	Same as for ammonium oxalate	Recrystallization from water
Sulfuric acid.....	Evaporate 10 cc. 10 N solution and ignite; test residue*		Distillation
Superoxol.....	Evaporate 10 cc. to dryness; test residue*		Vacuum distillation

* The test for calcium is as follows: To the residue left after evaporation or ignition add 1 drop of concentrated nitric acid and 3 cc. of water. Pour into a small conical centrifuge tube (15 cc. capacity) neutralize (to methyl red) with calcium-free ammonium hydroxide, add 2 cc. of 3 per cent ammonium oxalate solution let stand overnight, and centrifuge. No sediment should be detectable on careful examination of the tip of the centrifuge tube.

from unashed material, or from the slightly acid solution of the ash when dry ignition has been used. The most suitable receptacle for the precipitation is a large lipped Pyrex test-tube (200 X 25 mm.).

To the sample, diluted with water to a volume of about 10 cc., are added 2.5 cc. of 5 N ammonium chloride and 10 cc. of 2.5 per cent oxalic acid. The mixture is then gradually neutralized with approximately 5 N ammonium hydroxide to pH 5, the final adjustment being made if necessary with a few drops of the oxalic acid solution. The test-tube is shaken gently for 3 minutes after the calcium oxalate has begun to separate, and let stand overnight.

If the material available contains less than 0.25 mg. of calcium the precipitation must be conducted on a smaller scale. In this case water is added in sufficient quantity to bring the volume to 4 cc. (instead of 10), and the calcium is precipitated by adding 1 cc. of ammonium chloride, 4 cc. of oxalic acid, and the requisite amount of ammonium hydroxide. The procedure otherwise is that described above.

Filtration—On the following day the calcium oxalate is filtered off with suction through the small filtration tube described some years ago by one of us¹³ for handling magnesium ammonium phosphate precipitates. This consists of a glass tube, about 120 mm. long and of 8 mm. bore, flanged at one end and constricted at the other so as to leave a hole 2 mm. in diameter. As calcium oxalate precipitates may be finely divided, a tight filter is required. The filtration tube, which is supported by a rubber stopper in the neck of the suction flask, is provided at the lower (constricted) end with a mat of ashless paper pulp, made of paper of loose texture (e.g. Schleicher and Schüll No. 589, black ribbon) and 3 or 4 mm. thick, which must be well packed, especially at the outer edge. A piece of glass tubing, not less than 6 mm. in external diameter and cut off squarely at the lower end, may be used (as a pipette) for transferring the requisite amount of pulp to the filtration tube, as well as for packing down the mat, which should be so tight that the rate of filtration does not exceed 3 cc. per minute with the suction pump on full.

An equally effective filter, which may be removed more readily from the filtration tube and is likewise easier to burn, may be

¹³ Fiske, C. H., *J. Biol. Chem.*, **46**, 285 (1921).

made in the following way. A thin pulp mat (1.5 mm.) is first prepared in the manner just described. On top of this is placed a disc of ash-free filter paper (of the sort designed to retain fine precipitates), 8 mm. in diameter, cut out with a cork bore of the proper size. The disc is pressed down on the mat with the same glass tube that has been used to manipulate the paper pulp, or better with a glass rod of the same diameter which also is cut off squarely and not fire polished. The solid rod is likewise a more convenient instrument for transferring the paper disc to the bottom of the filtration tube.

The precipitate is washed with four 2 cc. portions of 3 per cent ammonium oxalate solution. The filtration tube, with its stopper, is then removed from the suction flask, and the tip rinsed off with water to remove any material that may have crept up the outside. The precipitate and the mat are transferred, through the hole in the lower end of the filtration tube, to a small platinum dish or crucible, with the aid of a sharpened nichrome wire and about 1 cc. of water. Any calcium oxalate still adhering to the glass is dissolved with 2 cc. of *N* nitric acid, added first to the test-tube, and then poured through the filtration tube into the platinum dish. The apparatus is finally rinsed, in the same order, with two 2 cc. portions of water.

Conversion to Calcium Oxide—The contents of the platinum dish are evaporated to dryness on the steam bath, leaving the calcium largely in the form of nitrate. The residue of calcium nitrate must now, before it is ignited, be reconverted to the oxalate.¹⁰ 0.5 cc. of 2.5 per cent oxalic acid solution (base-free) is consequently added, and the water is once more removed by evaporation on the steam bath. The dish is next supported on a triangle and heated with a micro burner flame, the temperature being gradually raised until the paper chars and finally begins to glow. At this stage the flame is removed until the glowing stops, and then applied at intervals until the paper is completely carbonized. The ignition is finished by heating the dish for a few seconds with the full flame of the micro burner.

Titration—As soon as the dish is cool enough to handle a drop of methyl red (saturated alcoholic solution) is added. If the dish has not become too cold the alcohol evaporates immediately, leaving the residue dyed yellow. 1 cc. of 0.02 *N* hydrochloric acid

is now introduced, and the dish rotated gently. Particles of calcium oxide remaining undissolved can readily be distinguished, by their yellow color, from immaterial traces of filter paper ash,

TABLE II

Determination of Calcium Alone and in Presence of Magnesium and Phosphate

Composition of solution			Volume	Ca present	Ca found
Calcium*	Magnesium†	Phosphorus‡			
mg. per cc.	mg. per cc.	mg. per cc.	cc.	mg.	mg.
0.03	0	0	10	0.300	0.300
0.03	0	0	10	0.300	0.298
0.02	0	0	50§	1.000	1.002
0.02	0	0	50	1.000	1.002
0.02	0	0	50	1.000	1.004
0.02	0	0	50	1.000	0.994
0.02	0	0	10	0.200	0.200
0.02	0	0	10	0.200	0.198
0.01	0	0	20	0.200	0.200
0.01	0	0	20	0.200	0.200
0.03	0.36	9	10	0.300	0.294
0.03	0.36	9	10	0.300	0.292
0.02	0.24	6	50§	1.000	1.018
0.02	0.24	6	50	1.000	1.014
0.02	0.24	6	10	0.200	0.202
0.02	0.24	6	10	0.200	0.200
0.02	0.24	0	50§	1.000	1.012

* The standard calcium solutions were prepared by dissolving Kahlbaum's calcium carbonate (zur Analyse) in nitric acid.

† Added as magnesium lactate which was prepared by neutralizing lactic acid with Merck's Blue Label magnesium oxide and recrystallizing from water. The salt contains 3 molecules of water of crystallization.

‡ Added as monopotassium phosphate (purified by recrystallization from water).

§ When the volume exceeds 20 or 25 cc. the precipitation is conducted in a lipped 100 cc. Erlenmeyer flask or conical beaker.

|| These analyses are included to show that precipitation may be incomplete in the presence of a large amount of phosphate if the mixture is shaken for only 3 minutes after precipitation has begun.

which cannot be brought into solution. In case 1 cc. of the standard acid does not suffice to dissolve the oxide readily, a second cc. is added and the dish manipulated until solution is complete. A small stirring rod may be used, but is unnecessary.

The excess of acid is titrated with 0.02 N sodium hydroxide from a micro burette to the full yellow color of the indicator, only 0.005 cc. being added at a time as the end-point is approached. 1 cc. of 0.02 N acid is equivalent to 0.4007 mg. of calcium.

Results—With the concentration of calcium within the limits of 0.01 to 0.03 mg. per cc., the method can be used, with an error not exceeding 1 per cent, for the analysis of mixtures containing, per

TABLE III

Determination of Calcium in Presence of Magnesium, Phosphate, and Sulfate

Composition of solution				Volume	Ca present	Ca found
Calcium*	Magnesium*	Phosphorus*	Sulfur†			
mg per cc	mg per cc	mg per cc	mg per cc	cc.	mg.	mg
0.02	0	0	8	10	0.200	0.200
0.02	0	0	8	10	0.200	0.202
0.02	0	0	16	10	0.200	0.200‡
0.02	0	0	16	10	0.200	0.202‡
0.03	0.36	9	12	10	0.300	0.300‡
0.03	0.36	9	12	10	0.300	0.298‡
0.02	0.24	3	8	10	0.200	0.202
0.02	0.24	3	8	10	0.200	0.200
0.02	0.24	6	16	10	0.200	0.200‡
0.02	0.24	6	16	10	0.200	0.202‡
0.01	0.24	6	8	20	0.200	0.202
0.01	0.24	6	8	20	0.200	0.202

* See Table II.

† Added as sulfuric acid. In these experiments the concentration of ammonium sulfate (after the acid had been neutralized with ammonium hydroxide) was 0.5 N or higher. The addition of ammonium chloride was therefore omitted.

‡ Shaken for 15 minutes after the calcium oxalate began to precipitate, and let stand overnight.

mg. of calcium, 12 mg. of magnesium, 200 mg. of phosphorus, and an even larger quantity of sulfur (Tables II and III). When the concentration of phosphorus exceeds 6 mg. per cc., or that of sulfur 8 mg. per cc., the calcium may not be completely precipitated if the directions given are adhered to. Under such conditions, however, the tendency to delayed precipitation is made evident by the fact that the calcium oxalate does not begin to separate at once. By shaking for 15 minutes (instead of 3 minutes) after the

first appearance of a precipitate, correct results can be obtained with even larger amounts of phosphorus and sulfur present (Table III).

Determination of Calcium in Human Urine

Digestion with sulfuric and nitric acids is a thoroughly satisfactory method for ashing urine provided that the amount of organic matter is not too large in proportion to the calcium, and may be used except when the calcium intake is very low or when the diet contains an excess of any substance (*e.g.* phosphate) that interferes with calcium absorption. Because ammonium sulfate tends to retard the precipitation of calcium oxalate, the amount of sulfuric acid that may be used with safety for this purpose is relatively small. Its effect on the precipitation will naturally be added to that of the sulfate already present in the urine. A very considerable amount of organic matter can eventually be destroyed by digestion with a little sulfuric acid (with the aid of nitric acid), but the analysis is likely to be spoiled by the formation of a precipitate—evidently metaphosphate—which will not dissolve in water. This difficulty will not infrequently arise in the case of urine obtained from experimental animals when the kidneys are excreting very little calcium, in which event a special method which permits destruction of the organic matter at a lower temperature must be used (*vide infra*). Ashing with sulfuric and nitric acids is on the other hand permissible with human urine of almost any kind, and is carried out as follows: A sample of urine containing between 0.25 and 0.75 mg. of calcium is boiled down in a large lipped Pyrex test-tube (200 × 25 mm.) with 1 cc. of 10 N sulfuric acid until white fumes appear. While the mixture is kept gently boiling, concentrated nitric acid, a drop or two at a time, is allowed to run down the wall of the test-tube, and the boiling is continued until most of the excess nitric acid has been driven off, as indicated by the reappearance of white fumes. This treatment is continued until the residue is colorless. When the tube has cooled, 10 cc. of water and 2.5 cc. of 5 N ammonium hydroxide are added, and the excess ammonia is neutralized with dilute nitric acid until the phosphate precipitate has just disappeared. 10 cc. of 2.5 per cent oxalic acid are now introduced, and the mixture is slowly neutralized to pH 5 with ammonium

hydroxide, as described above. The analysis is continued in the usual way.

Direct Precipitation—Calcium may also be precipitated from urine without ashing, following the directions given on p. 217. This method appears to give quite satisfactory results with ordinary human urine, but the character of the precipitate is such that the filter is soon clogged, and the analysis consumes more time than it would if the urine had been ashed. The filtration difficulty can be eliminated by heating the mixture for 15 minutes in a boiling water bath after the reagents have been added and the volume has been made up to 25 cc. In this way a considerable amount of time may be saved if a large number of samples must be analyzed

TABLE IV
Analysis of Human Urine

Each figure given is the average of duplicate analyses agreeing within 0.8 per cent or less.

Treatment	Sample 1	Sample 2
	mg. Ca per 100 cc.	mg. Ca per 100 cc.
Dry ashing.....	13.55	38.2
Ashed with 1 cc. 10 N H ₂ SO ₄ , with aid of HNO ₃ ...	13.53	37.8
Direct precipitation at room temperature	13.58	37.7
" " 15 min. in boiling water bath.....	13.52	37.9

at once, but the direct precipitation method (with heat) cannot be used unless the urine is quite free of material (other than calcium oxalate) which might be precipitated under the conditions used and carry down inorganic matter. Even traces of albumin, for example, interfere. The isoelectric point of the denatured urinary protein is not far from pH 5, and the protein coagulum is by no means free from ash. The urine must also be quite fresh, for bacteria contaminating the calcium oxalate precipitate will cause erroneous results.

All three of the procedures mentioned have been carried out on two samples of human urine, the calcium content of which had been determined by the dry ashing method. The results are identical throughout within less than 1 per cent (Table IV).

Special Method for Ashing Urine Low in Calcium

Whenever a sample of urine containing the requisite amount of calcium cannot be ashed easily with sulfuric and nitric acids, and particularly when the residue is not completely soluble in water, a more effective oxidizing agent must be used. Of the reagents tested for this purpose, including nitric, hydrochloric, and sulfuric acids, potassium chlorate, and hydrogen peroxide in various combinations, we have had the best success with a mixture of nitric acid and hydrogen peroxide (Merck's superoxol).

Nitric Acid-Superoxol Oxidation—The urine (containing between 0.1 and 0.3 mg. of calcium) is first evaporated to dryness in a large lipped Pyrex test-tube (200 × 25 mm.) by means of a current of heated air.¹⁴ The test-tube is then warmed by supporting it in a clamp about 4 cm. above the tip of a low micro burner flame, and the oxidation started by adding a few drops of concentrated nitric acid and 0.5 cc. of superoxol. The contents of the test-tube are in this way completely converted into foam. From this point on, until the last stage of the oxidation, the flame is applied only to the *sides* of the test-tube (which is partly filled with foam), and is kept in motion so that no spot is continuously heated for more than a fraction of a second. Meanwhile more superoxol is introduced, a few drops at a time, and in addition at intervals a drop or two of nitric acid. The contents of the tube should consist entirely of foam throughout this stage. In from 5 to 15 minutes the liquid should have become nearly colorless, indicating that most of the organic matter has been destroyed. At this point the heating is interrupted until the foam subsides, whereupon a few more drops of superoxol are added and the burner is returned to its former position, with the tip of the flame 4 cm. from the bottom of the test-tube. The heating is continued until the residue is entirely colorless, and the flame should be removed before the evolution of oxygen—from the residual hydrogen peroxide—

¹⁴ By means of apparatus which has been devised by Logan (Logan, M. A., *J. Biol. Chem.*, **86**, 761 (1930)) 40 cc. or more of urine, for example, can be evaporated in a test-tube of this size with almost no attention and without the slightest risk of loss by spattering or bumping. Evaporation, ashing, and precipitation may consequently all be carried out without transferring the material at any time.

has altogether ceased. This last phase of the oxidation takes about 1 minute.

The clear mass left at the bottom of the test-tube, when it has cooled, is dissolved with 3 cc. of water. The solution is made alkaline with 1 cc. of 5 N ammonium hydroxide, and the excess alkali just neutralized with N nitric acid, which is added drop by drop until the precipitate of phosphate redissolves. The calcium is precipitated in the customary manner by adding 4 cc. of 2.5 per cent oxalic acid and adjusting the reaction to pH 5 with ammonium hydroxide, enough water being added in the meantime so that the final volume will be approximately 10 cc. The rest of the process has already been described.

Double Precipitation Method

The urine of cats on a diet consisting solely of large amounts of meat may contain, per mg. of calcium, as much as 70 mg. of magnesium, 500 mg. of phosphorus, and 500 mg. of sulfur. The precipitation method outlined above is in this case useless, and presumably would not be reliable for the analysis of urine from any species under similar conditions. The following procedure, in which the calcium is first separated from most of the interfering substances, including the bulk of the organic matter, by a preliminary precipitation with oxalate and alcohol, gives satisfactory results.

An amount of urine containing from 0.2 to 0.4 mg. of calcium is transferred to a 100 cc. conical centrifuge tube and pure powdered oxalic acid (10 mg. for each cc. of urine) added. The oxalic acid is dissolved by rotating the tube and heating in a water bath, and the contents are then neutralized (to pH 5) with 5 N ammonium hydroxide. To precipitate the calcium, 0.5 volume of 95 per cent alcohol is now run in rapidly from a pipette, and the tube is rotated until the alcohol and urine are completely mixed. A very fine precipitate begins to appear almost at once.

After about 5 minutes the centrifuge tube is placed in a boiling water bath until the temperature of the contents has reached about 70°, when it is removed from the bath and rotated for 3 minutes.

On the following day the calcium oxalate is thrown down by centrifugation, and the supernatant fluid poured off. The sedi-

ment is dissolved and transferred to a large lipped Pyrex test-tube (200 × 25 mm.) with five 1 cc. portions of N nitric acid. 4 or 5 drops of 10 N sulfuric acid are added, and the mixture is boiled down and ashed. Since the amount of organic matter now present is very small the oxidation takes only a short time. The subsequent treatment of the residue is identical with that followed after nitric acid-superoxol oxidation (*vide supra*).

TABLE V

Analysis of 24 Hour Urines of Meat-Fed Cats by Double Precipitation Method

Ca, ashed	Ca, not ashed
mg.	mg.
0.52	0.54
0.87	0.86
1.12	1.11

TABLE VI

Analysis of Known Mixtures by Double Precipitation Method

The calcium salt was added to a mixture containing 22 mg. of magnesium (as lactate), 180 mg. of phosphorus (KH_2PO_4), 145 mg. of sulfur (added as H_2SO_4 and neutralized with NH_4OH), and 110 mg. of sodium chloride.

Ca present	Ca found
mg.	mg.
0.200	0.193
0.200	0.200
0.200*	0.200
0.400	0.400
0.400	0.402

* 2.6 gm. of sodium chloride were present.

The analyses in Table V show that this double precipitation method gives substantially the same results whether the urine has been ashed or not. Destruction of the organic matter may hence be omitted, except for the mild oxidation required to remove the organic impurities carried down with the first calcium oxalate precipitate. As a further check on the procedure a series of analyses has been run on known solutions containing from 0.2 to 0.4 mg. of calcium, 22 mg. of magnesium, 180 mg. of phosphorus, 145 mg. of sulfur, and 110 mg. or more of sodium chloride

(Table VI). Needless to say, the materials from which the salt mixtures were prepared were carefully purified to insure freedom from calcium. The magnesium lactate and the monopotassium phosphate were recrystallized from water, the sodium chloride was precipitated three times with calcium-free alcohol (see Table I), and the sulfuric acid was redistilled.

SUMMARY

The separation of calcium (as oxalate) in the presence of large amounts of phosphate, sulfate, and magnesium is discussed with special reference to the analysis of urine of low calcium content. Methods of effecting this separation under the most extreme conditions likely to be encountered in urine and other biological material are described, in conjunction with a procedure for the final analysis of the calcium oxalate precipitate, based on alkali-metric titration of the oxide.

DETERMINATION OF GLYCOGEN IN TISSUES*

BY MELVILLE SAHYUN

(From the Food Research Institute and the Department of Physiology, Stanford University, California)

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The purpose of the present paper is to present some simplifying modifications of Pflüger's method (1) for the estimation of glycogen, that render it available for small amounts of tissue. The suggested modifications involve (a) shortening of the time necessary to hydrolyze the tissue with strong alkali, (b) facilitation of the separation of the glycogen by precipitating it in the presence of charcoal and centrifuging, (c) hydrolysis of the separated glycogen for a shorter time than customary, and (d) the substitution of sulfuric acid for hydrochloric acid to avoid the introduction of chlorides.

(a) Less than the 2 hours of hydrolysis with strong potassium hydroxide recommended by Pflüger (1) has been found sufficient by Schöndorff and coworkers (2) who using 30 per cent potassium hydroxide found 30 minutes sufficient for liver and muscle, provided the flasks were shaken thoroughly every 5 to 10 minutes. Bierry and Gruzewska (3) obtained good results by autoclaving at 120° for 30 minutes with 35 per cent potassium hydroxide. The time recommended in the writer's procedure is 30 to 40 minutes.

(b) Pflüger (4) found the precipitation of glycogen by alcohol from its solutions in strong alkali may take place so slowly that there is danger of losses in its estimation. The procedure recommended in the present paper avoids this danger by adsorbing the glycogen on activated charcoal which is found not to interfere with the subsequent sugar determination. Moreover, when the washed glycogen is hydrolyzed, the filtration and clarification of

* This work is supported by a special grant from Mr. John Percival Jefferson, Santa Barbara, California.

the resulting sugar solution is facilitated. In lieu of filtering off the glycogen precipitated from alkaline solution, separation by centrifugation is advisable, a procedure suggested some years ago by Kahn (5). The presence of charcoal causes the complete deposition of glycogen at the bottom of the centrifuge tube.

(c) In accordance with the findings of Sahyun and Alsberg (6) hydrolysis with 1 N sulfuric acid for only 2 hours is necessary.

(d) By substituting sulfuric acid for hydrochloric acid any interference of chlorides with the Folin and Wu (7) sugar method is avoided. Everett, Shoemaker, and Sheppard (8) have found chlorides to interfere with the Folin and Wu (7) sugar determination while sulfates do not. Bischoff and Long (9) reported that they were unable to use the Folin and Wu sugar method for the estimation of muscle sugar owing to the rapid fading of the final blue color. It is interesting to note that their filtrates contained a considerable amount of potassium chloride which they had added to free them from protein. The writer also has observed that after hydrolysis with hydrochloric acid the blue color obtained upon the addition of the Folin and Wu reagent (phosphomolybdic acid) to the samples was unstable. Hydrolysis by Pflüger's method is accomplished by 2.2 per cent hydrochloric acid. This corresponds to about 0.58 N hydrochloric acid. Obviously when the solution is finally neutralized it must contain 0.58 N chloride salts. The data presented in Table IV show that concentrations as low as those actually occurring in the use of the Pflüger method are objectionable. The matter is of some interest in other connections. Herbert and Bourne (10) have investigated the reducing substances of blood other than glucose and observed that glutathione, which is present in the red corpuscles, plays an important rôle. To avoid its diffusion into the blood filtrate they suggested the addition of sodium sulfate and the use of half the usual amounts of $\frac{2}{3}$ N sulfuric acid and 10 per cent sodium tungstate. Folin and Svedberg (11) have simultaneously arrived at the same conclusion employing a different technique.

Reagents

Potassium Hydroxide—Two solutions of potassium hydroxide would prove helpful, one of 40 per cent strength, the other of 60 per cent. The 40 per cent solution is to be used for samples of 1

gm. or less; the 60 per cent solution for the larger samples. The object of this is to control the final volume after the precipitation of glycogen with alcohol.

Sulfuric Acid—Instead of hydrolyzing glycogen with the usual 2.2 per cent hydrochloric acid, sulfuric acid is to be used. A 2 N solution of sulfuric acid is required for the hydrolysis of glycogen.

Charcoal—Purified Merck medicinal charcoal¹ was used in our experiments. Three different samples of Merck charcoal were tested with the same results. The purpose of purifying is explained.¹

Method

1 gm. or less of tissues frozen in liquid air is introduced into a 15 cc. graduated centrifuge tube and enough 40 per cent potassium hydroxide is added to bring the mixture to the 5 cc. mark. If larger amounts of tissues are obtainable, to each gm. of the tissues used in a 50 cc. centrifuge tube, 1 cc. of 60 per cent potassium hydroxide is added so that the final volume of the alkali mixture does not exceed 10 or 15 cc. The tubes are covered with tin foil and placed in a boiling water bath for 30 or 40 minutes and shaken thoroughly every 5 or 10 minutes. About 50 mg. of active charcoal are then added and the contents well stirred with a fine glass rod, then twice the volume of ethyl alcohol is added to the alkali mixture, and the contents are thoroughly stirred once more. In case any glycogen sticks to the glass rod, it is washed with as small

¹ In Cole (Cole, S. W., Practical physiological chemistry, Cambridge, 8th edition, 469 (1928)) we find the following statement: "In the author's experience Merck Medicinal Charcoal, as an adsorbent, is far superior to any other preparation."

The writer finds that Merck medicinal charcoal contains organic impurities that possess slightly copper-reducing properties. The following treatment for its purification was found very effective. A quantity of Merck's medicinal charcoal is transferred to a beaker and covered with 1 N sulfuric acid. It is then boiled for 30 minutes, filtered while hot, and washed several times with boiling distilled water. The moist charcoal is finally transferred to a porcelain crucible and heated over a free flame for about 30 minutes to a dull red heat. Upon cooling, it is transferred to a clean bottle and stoppered.

It should be noted that heating is one of the standard methods for activating charcoal. The treatment recommended in effect changes the character of the charcoal and virtually makes a new preparation of it.

an amount of hot water as possible into the tube and twice that volume of alcohol is added. The tubes are centrifuged for 10 minutes. The glycogen is adsorbed completely on the charcoal and collects at the bottom of the centrifuge tube. The alkali-alcohol supernatant liquid is discarded. 5 cc. of hot distilled water or less are added along with a strip of litmus paper. A few drops of 2 N sulfuric acid are added to turn the blue litmus paper red and followed by an equal volume of acid of this concentration. The centrifuge tubes are then replaced for 2 hours in the boiling water bath whereupon hydrolysis is complete. The contents are then neutralized with 1 N or 2 N sodium hydroxide and introduced quantitatively into volumetric flasks.² After the mixture is cooled, diluted to the mark, and mixed thoroughly, samples are removed and filtered. The filtrate is clear and colorless and ready for sugar determination.

In testing the method, small amounts of glycogen were added to tissues free from glycogen. Liver tissues were used throughout this experiment. Several glycogen solutions of various concentrations were prepared from one of our glycogen preparations described in an earlier paper (12). The glycogen solutions were carefully measured and mixed with the tissues. Duplicate samples of pure glycogen were set aside in each case for the determination of the theoretical amount. The results are recorded in Table I. The reducing sugar was determined by the method of Folin and Wu (7). The corresponding values in terms of glycogen were obtained by multiplying the value obtained for glucose by the factor 0.927.³ The blue color formed upon the addition of the phosphomolybdic acid reagent was not observed to suffer any change in intensity upon 30 minutes standing.

The method was used for the determination of unknown amounts of glycogen in the tissues of rabbits and dogs. In certain instances liquid air was used. This is indicated in Table II. In

² When one is dealing with samples of less than 1 gm., the procedure at this stage in this laboratory is to dilute the samples to the 10 or 15 cc. mark and centrifuge for 3 minutes. The impurities present separate out very easily.

³ This factor has been questioned by Nerking (13) and by Kerly (14). Both have assigned to it a value approaching 0.95. It seems desirable, however, to continue using the factor 0.927 until this question is definitely settled.

so far as possible duplicate samples were determined. Our object in this experiment is not to discuss the amount of glycogen found

TABLE I

Determination of Glycogen Added to Glycogen-Free Tissues According to Method Described

Determination No	Glycogen found in tissues	Theoretical amount of glycogen added	Determination No	Glycogen found in tissues	Theoretical amount of glycogen added
	mg.	mg.		mg.	mg.
1	5.2	5.0	11	48.7	48.0
2	5.5	5.0	12	62.7	64.0
3	10.0	10.0	13	77.0	80.0
4	10.0	10.0	14	153.0	160.0
5	10.0	10.0	15	78.8	80.0
6	15.0	15.0	16	79.3	80.0
7	14.8	15.0	17	30.1	30.0
8	19.8	20.0	18	60.4	60.0
9	32.0	32.0	19	89.5	90.0
10	16.7	16.0	20	88.5	90.0

TABLE II

Glycogen Content of Various Muscles with and without the Use of Liquid Air

Skeletal muscle	Weight of sample	Liquid air	Glycogen content per 100 gm.
	gm.		gm.
Left gracilis (dog).....	4.7	Yes	1.05
Duplicate	4.2	"	1.05
Right rectus (dog).....	3.0	"	1.30
Left gastrocnemius (dog)...	6.7	No	1.09
Duplicate.	5.2	Yes	1.01
"	5.05	"	1.02
Right rectus (rabbit).....	5.2	No	0.229
Duplicate.	5.2	"	0.231
Left rectus (rabbit).....	6.0	"	0.220
Duplicate.	7.5	"	0.225
Right gracilis (rabbit)....	5.1	"	0.207
Duplicate.	5.2	"	0.204
Left gracilis (rabbit).....	5.35	"	0.207
Duplicate.	6.35	"	0.205

in various types of skeletal muscles, but to test the method. To determine that the addition of charcoal introduces no errors,

known amounts of glucose solutions were carefully measured and mixed with the activated charcoal in test-tubes which were then placed in a boiling water bath for 5 minutes. Their contents were filtered and the filter washed with hot water. The filtrates were collected in volumetric flasks, cooled, and made up to volume. The results are given in Table III. Similar results were obtained with different samples of Merck's charcoal. Mr. Paine of the Bureau of Chemistry and Soils very kindly furnished four samples of commercial adsorbent charcoals: carboraffin, norit, suchar, and darco. With the exception of darco all gave satisfactory

TABLE III

Determination of Known Amounts of Pure Glucose in Solution to Which Activated Charcoal Had Been Added

The solutions were filtered and the amounts found are compared with glucose solutions of identical strength to which no charcoal had been added.

Determination No.	Amount of glucose found (charcoal)	Amount of glucose found (no charcoal)
	<i>mg.</i>	<i>mg</i>
1	5.0	5.0
2	9.8	10.0
3	20.0	20.0
4	25.0	25.0
5	29.5	30.0
6	40.0	40.0
7	50.0	50.0

results. These charcoals were used as received without preliminary heating or washing.

To demonstrate the effect of the presence of chloride on the Folin and Wu (7) sugar method, the following experiments are presented.

A solution of pure glucose was prepared so that 100 cc. contained 100 mg. From this stock solution samples were introduced into volumetric flasks to which the desired concentration of sodium chloride was added and which were then made up to volume with distilled water. Each experiment was repeated several times and not more than four samples were determined at one time. About 5 minutes were required to adjust the standard and complete the first set of readings. No sodium chloride was added to the stand-

ard. The Folin and Wu macro sugar method was used. The final blue color was compared with a Leitz-Wetzlar colorimeter. The first set of readings was recorded in each instance 5 minutes after the addition of the phosphomolybdic acid reagent, dilution to the mark, and mixing. The results are given in Table IV.

This experiment confirms the findings of Everett and coworkers (8), that chloride ions interfere with the development of the final blue color when reducing substances are determined by the above method.

The influence of sodium sulfate on the determination of sugar

TABLE IV

Effect of Sodium Chloride on the Determination of Sugar According to the Method of Folin and Wu, Showing the Variations in Mg. per 100 Cc.

NaCl concentration per liter	After		
	1 to 5 min.	10 to 15 min.	20 to 25 min.
<i>M</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
Standard (none)	100	100	100
1.0	65	42	
0.8	70	52	45
0.6	78	60	50
0.5	84	66	55
0.4	91	77	67
0.3	95	80	71
0.2	96	89	83
0.1	98	91	89

Experiments with potassium chloride were performed and showed it to possess a similar effect.

by the Folin and Wu method was studied in a manner similar to the previous experiment. Several sugar solutions in various concentrations of sodium sulfate, ranging from 1 N to 0.01 N were investigated. No change was observed in the final blue color for 40 minutes except in solutions containing 1 N sodium sulfate. In fact, in concentrations of 0.6 N sodium sulfate there was a stabilization of the final blue color. It was therefore found convenient to prepare the alkaline copper reagent of Folin and Wu in 0.6 N sodium sulfate. The modified reagent has been standing in this laboratory for 4 months without deterioration.

I wish to express my thanks to Dr. C. L. Alsberg for valuable suggestions.

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STUDIES ON GLYCOGEN

THE HYDROLYSIS OF GLYCOGEN IN VARIOUS CONCENTRATIONS OF ACIDS, AND THE HYDROLYSIS OF GLYCOGEN WITH TAKADIASTASE

BY MELVILLE SAHYUN AND CARL L. ALSBERG

(From the Food Research Institute and the Department of Chemistry, Stanford University, California)

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INTRODUCTION

In developing methods for the estimation of glycogen, many investigators have studied its hydrolysis with acids for the purpose of ascertaining the conditions that insure as nearly complete inversion as possible (1). The general agreement finally reached is that the best condition is boiling with 2.2 per cent hydrochloric acid for 3 to 5 hours. Sulfuric acid was found less satisfactory although Nerking (2) obtained good results with it; phosphoric and citric acids were found too weak. In Part I of the present paper this question is reinvestigated for the purpose of furnishing, possibly, a basis for shortening and improving the customary method of estimating glycogen. In addition a study of the kinetics of the reaction is presented, for it does not seem to have been investigated previously. Pringsheim and Will (3) present a few incidental data on the rate of hydrolysis by pancreatic amylase. Paechtner (4) reached the conclusion that hydrolysis by salivary diastase is a so called monomolecular reaction. He studied the rate of disappearance of opalescence by means of a dipping refractometer. His conclusion, therefore, is applicable only to the first stage of the reaction, for, as is shown in the present paper, glycogen may lose its opalescence when hydrolyzed by acid to a slight degree without loss of its other principal characteristics. The kinetics of the hydrolysis of glycogen, whether monomolecular or of some higher order, might indicate whether the ultimate products of hydrolysis consist of a single or of more than one saccharide and

whether intermediary products are formed during the course of the reaction. These questions are of special interest now that Laquer and Meyer (5) have presented evidence that some as yet unknown carbohydrate is an intermediate product between glycogen and lactic acid in muscle and since Barbour (6) has reported the formation of a trisaccharide through the action of muscle glycogenase on glycogen. This trisaccharide he assumed to have a specific rotation of $+182^\circ$.

Part II of this paper deals with the hydrolysis of glycogen with takadiastase with special reference to the equilibrium reached and

TABLE I
Hydrolysis of Glycogen in Various Concentrations of Hydrochloric and Sulfuric Acids

50 mg. of glycogen were used in each case.

Concentration of acid	Glucose formed	
	HCl	H ₂ SO ₄
<i>N</i>	<i>mg.</i>	<i>mg.</i>
0.01	1.5	1.5
0.05	7.5	6.0
0.10	18.0	12.0
0.20	36.0	25.0
0.30	45.0	36.0
0.40	48.0	41.0
0.50	50.0	44.0
0.60	52.0	45.0
0.70	52.0	48.0
0.80	52.0	52.0
1.00	52.0	52.0

the effects of the presence of various sugars on the action of this enzyme.

Part I. Hydrolysis of Glycogen in Various Concentrations of Acids

The glycogen used in these experiments was prepared from rabbit livers according to the method of Sahyun and Alsberg (7). A glycogen solution was freshly prepared for each series of experiments. Its strength is given in each case. In preparing a 1 per cent solution, for example, 1 gm. of glycogen was accurately weighed in a 50 cc. beaker and dissolved in boiling water. It was

then transferred quantitatively to a 100 cc. volumetric flask and hot distilled water was added, cooled, and made up to the mark.

In preparing the desired concentration of an acid mixture, a 2 N solution was prepared and used through the various experiments after appropriate dilution. For concentrations greater than 1 N a 10 N solution was kept on hand. Thus to obtain a 1 N hydrochloric acid-glycogen mixture the desired amount of glycogen was transferred from the 1 per cent stock solution of glycogen to a Pyrex test-tube (capacity 50 cc.) and an equal volume of 2 N hydrochloric acid was added. After hydrolysis the solution was neutralized with 1 N sodium hydroxide and the content of the test-tube transferred quantitatively to a 50 cc. or 100 cc. volumetric flask.

The Folin and Wu (8) sugar method was used. All determinations were made in duplicate.

Experiment 1—In this experiment the hydrolysis of glycogen with various concentrations of hydrochloric and sulfuric acids was studied. The test-tubes were capped with lead foil and placed in a boiling water bath for about 3 hours. The results are given in Table I.

Hydrolysis of Glycogen with Normal Solutions of Hydrochloric and Sulfuric Acids

One object of these experiments, aside from the substitution of another acid for hydrochloric acid was to determine whether the time required for the quantitative determination of glycogen could be shortened. Obviously the acid should not cause any chemical change in the ultimate product of hydrolysis. In the following experiments, special attention was given to the appearance of reducing sugars at successive intervals of time as indicated in Table II. Heating with the acids was continued for some time after hydrolysis had been completed and sugars were then determined in order to learn whether the acids caused any destruction of the ultimate product of hydrolysis.

Experiment 2—10 cc. of 1 per cent glycogen were introduced into a 50 cc. volumetric flask along with 15 cc. of distilled water. 2 N hydrochloric or sulfuric acid was then added to bring the content to the mark. The contents were rapidly mixed, transferred to a 100 cc. Erlenmeyer flask, and immersed in the boiling water bath.

The time was taken from the moment when the samples were immersed in the bath. The results are given in Table II.

Analogous experiments were carried out with phosphoric acid. Hydrolysis was incomplete in all strengths of acid less than 1 N.

Kinetics of the Reaction

In determining the rate of glycogen hydrolysis, it was found desirable to use concentrations of acids weaker than 1 N.

TABLE II
Rate of Hydrolysis of 100 Mg. of Glycogen in 1 N Hydrochloric and 1 N Sulfuric Acid

Time	Glucose formed	
	N HCl	N H ₂ SO ₄
<i>min.</i>	<i>mg.</i>	<i>mg.</i>
10	25	10.5
20	65	35.5
30	93.5	57.5
40	95	75.5
50	97.5	85.5
60	97.5	95.0
75	100.0	100.0
90	105.0	105.0
105	105.0	105.0
120	105.0	105.0
180	105.0	105.0

Experiment 3—20 cc. of 1 per cent glycogen solution (200 mg.) were introduced into a 100 cc. volumetric flask along with 30 cc. of distilled water. 50 cc. of 1 N hydrochloric acid were then added, the contents thoroughly mixed, and rapidly transferred to a 250 cc. Erlenmeyer flask in which a thermometer had been inserted. The flask was then immersed in a boiling water bath. The time when the thermometer registered 98° was regarded as zero time. The temperature of 99° remained constant throughout the experiment. Samples were removed at successive intervals of time as indicated in Table III. The samples removed were first cooled and then neutralized with 0.5 N sodium hydroxide.

K was calculated in the monomolecular equation:

$$K = \frac{l}{t} \ln \frac{a}{a-x}$$

The values for K are recorded in the last column of Table III. The values for x in the table are computed by subtracting the amount of reducing sugar that appeared at zero time from each of the amounts of the reducing sugars actually determined.

TABLE III

Hydrolysis of 200 Mg. of Glycogen in 0.5-N Hydrochloric Acid Showing the Applicability of the Monomolecular Equation

$$K = \frac{l}{t} \ln \frac{a}{a-x}$$

Time	Hydrolyzed glycogen as glucose	True values of reducing sugars	$a - x$	K
<i>min.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
0	5.0	00.0	213.0	
10	56.0	51.0	162.0	0.027
20	100.0	95.0	118.0	0.030
30	134.0	129.0	84.0	0.031
40	152.5	147.5	65.5	0.030
50	163.5	158.5	54.5	0.028
60	174.0	169.0	44.0	0.027
75	183.0	178.0	35.0	0.024
90	191.0	186.0	27.0	0.024
105	200.0	195.0	18.0	0.024
150	218.0	213.0	00.0	
180	218.0	213.0	00.0	

Otherwise the amount determined at the end of the first 10 minutes would not represent the true value of the hydrolyzed glycogen during that period of time.

Hydrolysis of Glycogen in 0.5 N Sulfuric Acid

The rate of hydrolysis of glycogen in 0.5 N sulfuric acid shows even more consistent values for K (Table IV) than hydrolysis with hydrochloric acid (Table III). Because sulfuric acid is the weaker of the two acids, the hydrolysis of glycogen proceeds at a slower rate so that experimental errors are appreciably reduced.

TABLE IV

Hydrolysis of 200 Mg. of Glycogen in 0.5 N Sulfuric Acid

This table shows the applicability of the monomolecular equation.

Time	Hydrolyzed glycogen estimated as glucose	$a - x$	K
<i>min.</i>	<i>mg.</i>	<i>mg.</i>	
0	00.0	220.0	
10	23.0	197.0	0.0113
20	44.5	175.5	0.0111
30	62.5	157.5	0.0112
40	82.0	138.0	0.0115
50	95.0	125.0	0.0113
60	108.0	112.0	0.0112
75	124.0	96.0	0.0110
90	140.0	80.0	0.0112
105	153.0	67.0	0.0110
120	167.0	53.0	0.0118
135	176.0	44.0	0.0119
187	196.0	24.0	0.0118
200	200.0	20.0	0.0119
230	220.0	00.0	

The above experiment was repeated in the presence of 200 mg. of glucose. Almost identical values for K were obtained.

TABLE V

Estimation of Specific Rotatory Power of the Product of Hydrolysis of Glycogen with Acid

Sample	Reducing sugar	α	$[\alpha]_D$
	<i>gm. per 100 cc.</i>	<i>degrees</i>	<i>degrees</i>
A	1.46	+1.70	+52.9
B	1.46	+1.69	+52.6

After standing overnight

A	1.43	+1.67	+52.4
B	1.38	+1.63	+52.9

Experiment 4—This experiment is identical with Experiment 3 except that sulfuric acid was substituted for hydrochloric acid. It is to be noted, however, that no measurable amount of reducing sugars appeared at zero time. The results are as recorded in Table IV and graphically in Fig. 1.

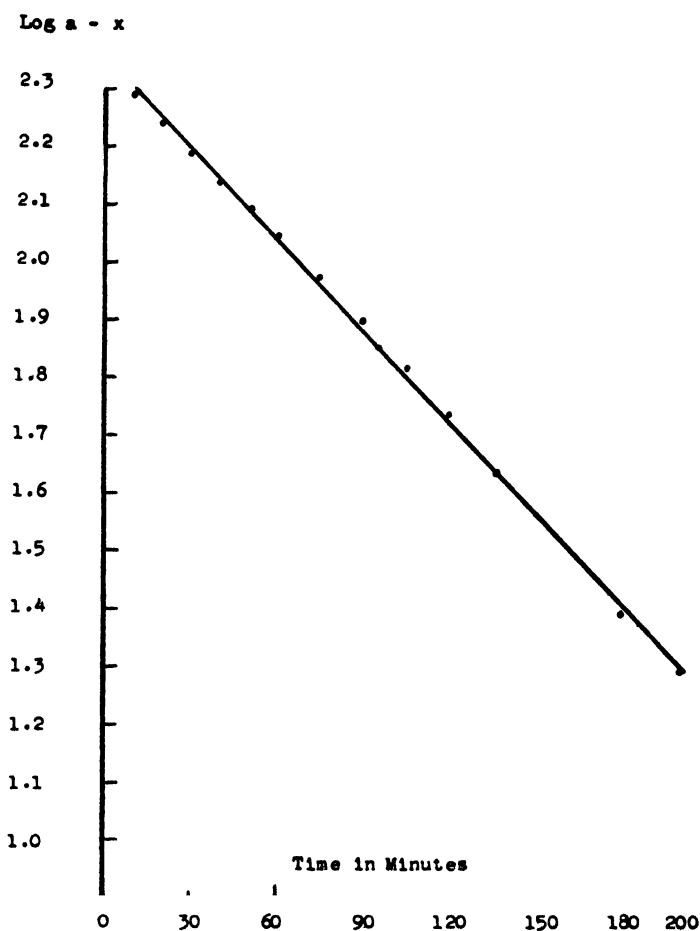


FIG. 1. This graph shows that the hydrolysis of glycogen with sulfuric acid proceeds like a pseudomonomolecular reaction.

Specific Rotatory Power of Products of Hydrolysis of Glycogen with Acid

Experiment 5—The glycogen used was as pure as that used in the preceding experiments, except that it was contaminated with minute traces of charcoal used in its preparation that could not be removed by filtration. Two samples, each weighing about 1.5 gm., were completely hydrolyzed in 1 N hydrochloric and 1 N

sulfuric acid respectively, cooled, transferred to volumetric flasks, and made up to volume. Since the hydrolysates were not quite clear and colorless, they were made quite clear and colorless by filtering twice through active charcoal. Samples were then removed for sugar determinations and the specific rotatory power of the respective solutions was then estimated. The values for α recorded below were the averages of ten readings in each case. The solutions were allowed to stand overnight, when their specific rotations as well as the amount of reducing substances were once more estimated.

In Sample A (Table V) hydrochloric acid was used; in Sample B sulfuric acid.

Fractionation of Products of Partial Hydrolysis of Glycogen with Acid by Ultrafiltration

When glycogen is acted upon by an acid, gradual disappearance of opalescence may be observed until it presents to the naked eye the appearance of a non-colloidal solution. The disappearance of opalescence is not an index of the absence of glycogen, for the clear solution gives the usual iodine color reaction.

Experiment 6—In a Pyrex test-tube 1 gm. of glycogen was dissolved in 0.1 N sulfuric acid and placed in a boiling water bath until the opalescence had completely disappeared, and was then kept there for another 20 minutes. The test-tube was then removed; its contents were neutralized with N sodium hydroxide, transferred to a 50 cc. volumetric flask, and made up to volume. The contents of the flask were then placed in an ultrafilter (cellophane membrane No. 600 being used). Ultrafiltration was allowed to proceed overnight. The volume of the ultrafiltrates was approximately 50 cc.

The non-ultrafiltrable fraction in the ultrafilter was dissolved in the minimum amount of hot water and solid potassium hydroxide was added to bring about a concentration of 40 per cent potassium hydroxide. The alkaline mixture was placed in a boiling water bath for 1 hour. Upon the addition of twice its volume of alcohol a precipitate was formed which was centrifuged, redissolved, and reprecipitated. The final product was then taken up in 100 cc. of distilled water. The rotatory power of the solution

was determined at room temperature. Samples were also hydrolyzed for sugar determination. The results were as follows:

Non-Ultrfiltrable Fraction

After hydrolysis, reducing substances es- timated as glucose	Equivalent of reduc- ing sugar as glycogen	α	$[\alpha]_D$
mg.	mg.	degrees	degrees
600	550	+2.36	+196

The specific rotatory power $+196^\circ$, found for the material precipitated by alcohol, is based on the assumption that the factor 0.927 is correct for the conversion of the glucose value found on acid hydrolysis into the corresponding polysaccharide. The validity of this factor under these circumstances has not been investigated by the writers.'

The ultrafiltrates were analyzed for reducing sugars. Samples were then hydrolyzed in 1 N sulfuric acid and the reducing substances redetermined. Before hydrolysis the reducing substances amounted to 75 mg. After hydrolysis of the ultrafiltrates the total reducing sugars amounted to 265 mg.

The disappearance of opalescence is the first striking change observed when such acids as were studied in this investigation act on glycogen. This change is not accompanied by the complete disappearance of glycogen. When weaker concentrations of either hydrochloric or sulfuric acid were used instead of 1 N or 0.5 N solutions, as in Experiment 6, the acid-glycogen mixture could be removed from the water bath, cooled, and neutralized before the appearance of but slight amounts of reducing substances. This in fact occurred in Experiment 4, in which, as the temperature reached 99° , the opalescence had almost disappeared while the samples that were removed and analyzed for reducing sugar showed no traces of it. This point was investigated further in the preceding experiment, Experiment 6. The glycogen-acid mixture was allowed to stand for a considerable period of time in the boiling water bath, even after the disappearance of opalescence. Analysis showed that about 10 per cent of the total amount of glycogen had been converted into reducing sugars. Upon neutralization the glycogen could be separated by precipitation with alcohol. Though the glycogen separated in this manner loses its opalescence

it yet retains most of its other properties. It gives the same color reaction with iodine as true glycogen. Strong potassium hydroxide has no apparent effect upon it. It is not ultrafiltrable. It can be precipitated with 66 per cent alcohol and the precipitate is soluble in water.

When the ultrafiltrates (Experiment 6) were treated with boiling potassium hydroxide, the clear colorless liquid containing but little reducing sugars changed to brilliant yellow. This is not an indication that substances other than a simple monosaccharide were present in the ultrafiltrates. On the other hand, when samples of the ultrafiltrates were hydrolyzed with acids, the amount of reducing sugars was considerably increased. It becomes obvious that an ultrafiltrable polysaccharide is an intermediate product between the non-opalescent glycogen and glucose.

The specific rotatory power of the non-opalescent glycogen was studied. Its separation cannot be accomplished by mere precipitation from the mixture since intermediate products are likewise precipitated. Ultrafiltration is therefore more suitable.

Conclusion

One can safely conclude from the experiments presented that sulfuric acid can be substituted for hydrochloric acid in the hydrolysis of glycogen. Since sulfates do not interfere with the color reaction of the colorimetric sugar method, as has been reported by Folin (9) and as has been observed by us, the Folin and Wu sugar method becomes more reliable. The substitution of sulfuric acid for hydrochloric acid is therefore recommended.

In the experiment dealing with the specific rotation of the product of glycogen hydrolysis, it is shown that the value for $[\alpha]_D$, related to the reducing power of the solution in terms of glucose, is that of glucose, $+52.5^\circ$. If any other substance is formed, it must therefore be one without optical activity and without reducing power, unless it is one that is adsorbed by charcoal or formed in very minute amounts. Furthermore, that there is formed an appreciable amount of a reducing substance other than glucose, which is adsorbed by charcoal, is rendered improbable by the observation of Sahyun (10) that in the presence of charcoal glycogen can be estimated accurately from the reducing power after acid hydrolysis. It is, however, not impossible that

in addition to glucose a phosphoric acid ester may be formed, but the amount can only be small, since rabbit liver glycogen contains less than 0.05 per cent of phosphorus.

Though the evidence presented in this paper tends to show that the rate of hydrolysis of glycogen with acids follows that of a pseudomonomolecular reaction, yet the conclusion cannot be drawn that glucose is derived directly from glycogen.

The action of sulfuric or of hydrochloric acid on glycogen can therefore be summarized as follows: The particle of glycogen is probably first reduced in size so that a non-opalescent glycogen is formed. The latter gives rise to one or more ultrafiltrable polysaccharides which are the direct source of the reducing sugar, glucose, the ultimate product of acid hydrolysis of glycogen.

Part II. Hydrolysis of Glycogen with Takadiastase

According to Norris (11) the rate of hydrolysis of glycogen by pancreatic diastase is at first very rapid, the glycogen being quickly converted into dextrans with simultaneous production of maltose. The further breakdown of these dextrans takes place with extreme slowness and is, as a rule, incomplete. Pringsheim and Will (3), however, obtained 74.8 per cent hydrolysis in 72 hours and 92 to 94 per cent in 48 hours if yeast coenzymes were added. Rona and Van Eweyk (12) investigated the action of salivary amylase and confirmed Norris's findings. Reference has already been made to the work of Paechtner. Barbour (6), using muscle glycogenase, reported that apparently the sole product of the hydrolysis of glycogen is a trisaccharide. With the exception of Barbour, no attempt was made by any of the above investigators to isolate or identify the product of hydrolysis.

Since little is known about the products of glycogen hydrolysis by enzymes, it was considered worth while to study the action of takadiastase on glycogen prepared from rabbit livers. The takadiastase used in this investigation was observed to contain a considerable amount of organic and inorganic impurities. An attempt was therefore made to purify the enzyme by ultrafiltration as described in a later paragraph.

The hydrolysis of glycogen with takadiastase follows the general action of enzymes on similar complex substances. Its action at first proceeds rapidly and gradually it becomes slower until at the

end of a certain time an equilibrium is reached between the substrate and the products of hydrolysis. It is shown by the following experiment.

Experiment 1—500 mg. of pure glycogen were dissolved in about 100 cc. of boiling water and introduced into a 250 cc. volumetric flask and cooled. 50 mg. of takadiastase were introduced along with distilled water to bring the volume up to the mark. The flask was then shaken several times together with some toluene and a sample was immediately removed and placed in boiling water

TABLE VI

Action of 50 Mg. of Takadiastase on 500 Mg. of Glycogen in a 250 Cc. Volumetric Flask at T 38°

Time		Appearance of reducing sugars, net amount	Time		Appearance of reducing sugars, net amount
hrs.	min.	mg.	hrs.	min.	mg.
0	0	00.0	3	00	82.0
	20	14.5	4	00	90.0
	40	29.5	5	00	97.0
1	00	39.0	6	20	112.0
1	20	42.5	7	00	118.0
1	40	55.5	8	00	125.0
2	00	59.0	24	00	280.0
2	30	76.0	30	00	280.0

$$280 \text{ mg.} \times 0.927 = 260 \text{ mg. glycogen; } \frac{260}{500} = 52 \text{ per cent.}$$

The initial amount of reducing substances in 50 mg. of takadiastase was found to be 16.5 mg. Ten subsequent determinations were made at various intervals of time that yielded the same amount.

for 5 minutes. The 250 cc. flask was then placed in a water bath kept at the constant temperature of 38°. Samples were removed as indicated in Table VI. The addition of sodium tungstate and $\frac{2}{3}$ N sulfuric acid in the proportion that one would add to precipitate blood proteins was found very efficient in stopping the further action of the enzyme in the samples to be analyzed. As a control 50 mg. of takadiastase dissolved in water were put in a 100 cc. volumetric flask and placed in the water bath at the same time. The reducing substances in this sample were found to be the same during the experiment and equal to the initial amount determined

at zero time. This value was therefore subtracted to give the net product of hydrolysis. The results are given in Table VI.

Ultrafiltration of Takadiastase

It has been observed by Truskowski (13) that the adsorption of diastase on foreign matters appreciably retards the action of this enzyme on the substrate. The takadiastase used in our experiments is a commercial preparation and was found to contain a considerable amount of impurities which we have succeeded in

TABLE VII
Hydrolysis of Glycogen with Crude and with Ultrafiltered Takadiastase

Time	100 mg. crude enzyme*		10 cc. ultrafiltered fraction		10 cc. ultrafiltrate and 10 cc. ultrafiltered fraction		10 cc. ultrafiltrate	
	Mg. reducing substances found							
	Total	Net	Total	Net	Total	Net	Total	Net
hrs.								
0	34	0	5	0	42	0	42	0
1.5	40	6	21.5	16.5	43	1	42	0
3	50	16	30	25	52	10	43	1
6	62	28	39	34	56	14	42	0
12	68	34	46.5	41.5	64	22	42	0
24	85	51	55	50	68	26	42	0

Hydrolysis was carried on at room temperature, about 23°.

* In this, the opalescence of glycogen disappeared almost completely after 24 hours; while in the remaining three flasks the opalescence did not disappear.

removing to a considerable extent. In a former paper (7) we commented on the high phosphorus content of this particular preparation. 90 per cent of the phosphorus along with 80 to 90 per cent of the reducing substances and most electrolytes and coloring matters were found to be easily removed by ultrafiltration through a cellophane membrane in an ultrafilter at a high nitrogen pressure.

Experiment 2—1 gm. of takadiastase was taken up in 100 cc. of water and ultrafiltered. Cellophane membrane No. 600 was used. The ultrafiltrates were collected and the non-ultrafiltrable fraction

was taken up again in the original amount of water. The non-ultrafiltrable fraction of the enzyme (designated in the tables as ultrafiltered fraction), the ultrafiltrate, or a mixture of the two was introduced into different flasks containing 10 cc. of a 1 per cent glycogen solution. The control contained crude takadiastase and glycogen. The final volumes were 100 cc. The rates of hydrolysis under these conditions are given in Table VII.

TABLE VIII

Effect of Ultrafiltrable Fraction of Takadiastase on the Rate of Glycogen Hydrolysis by Crude Takadiastase and by the Active Non-Ultrafiltrable Fraction of the Enzyme

Time	20 mg crude enzyme		10 cc. ultrafiltered fraction*		5 cc ultrafiltrate		5 cc ultrafiltrate and 10 cc ultrafiltered fraction		20 mg crude enzyme and 5 cc. ultrafiltrate	
	Mg reducing substances found									
	Total	Net	Total	Net	Total	Net	Total	Net	Total	Net
<i>hrs</i>										
0	7	0	0	0	12.5	0	12.7	0	19.5	0.0
1.5	19	12	7	7	12.9	0.4	20.0	7.3	28.8	9.3
3	21.8	14.8	9	9	12.6	0.1	23.5	10.8	32	12.5
4.5	23.5	16.5	15	15	12.9	0.4	28.5	15.8	38	18.5
7	30	23	18.5	18.5	12.5	0	30	17.3	40	20.5
24	55	48	33.5	33.5	12.5	0	46	33.3	67	47.5
30	56	49	36.5	36.5			50	37.5	69.5	50.0

* It is to be remembered that in this experiment in which glycogen was acted upon by the non-ultrafiltrable fraction of the enzyme, the amount used corresponds to only 10 mg.; whereas in the control 20 mg. of crude takadiastase were used.

Experiment 3—The preceding experiment was repeated, smaller quantities of the enzyme being used in each case. 500 mg. of takadiastase were ultrafiltered as previously. The volume of the ultrafiltrate amounted to 100 cc. The non-ultrafiltrable fraction was taken up in 500 cc. of distilled water. As in the preceding experiment, an initial determination of the reducing substances was made. Each flask contained 100 mg. of glycogen. The total amounts of reducing sugars as well as the net amounts formed by hydrolysis are given in Table VIII.

TABLE IX

Influence of Various Sugars on the Rate of Hydrolysis of Glycogen by Crude Takadiastase

Time	Control		Galactose		Dextrose		Levulose*	
	Mg. reducing substances found							
	Total	Net	Total	Net	Total	Net	Total	Net
<i>hrs.</i>								
0	7.5	0	103	0	100	0	100	0
2.75	19.0	11.5	81.5	-21.5	100	0	114	14
5.5	28.5	21.0	80.0	-23.0			121	21
8.5	42.5	35.0	100	-3	117	17	135	35
22	48.5	41.0	114	11	125	25	140	40
24	60	52.5	137	34	133	33	149	49

* The flask which contained levulose in addition to glycogen and takadiastase was not opalescent by the end of the experiment, contrary to all the other flasks of this series. The complete disappearance of opalescence was observed to take place between 8 and 9 hours after the addition of levulose.

TABLE X

Influence of Various Combinations of Sugars on the Rate of Hydrolysis of Glycogen by Crude Takadiastase

Time	Control		Dextrose, galactose, and levulose*		Dextrose and galactose	
	Mg. reducing substances found					
	Total	Net	Total	Net	Total	Net
<i>hrs.</i>						
0	7.5	0	261	0	164	0
4.25	23.5	16	261	0	164	0
7	31.5	24	224	-37	145	-19
10	41	33.5	256	-5	169	5
24	56.5	49	265	4	170	6
28	57.5	50	260	-1	170	6

* Opalescence disappeared completely before the end of the experiment.

Influence of Various Sugars on the Action of Takadiastase on Glycogen

In this and the following series of experiments a study was made of the influence of various sugars on the hydrolysis of glycogen with takadiastase.

Experiment 4—Each flask contained 100 mg. of glycogen and

20 mg. of crude takadiastase in a volume of 100 cc., and, except in the controls, either 100 mg. of *crystalline* galactose, *crystalline*

TABLE XI
Influence of Various Sugars on Crude and Ultrafiltered Takadiastase in Presence of Glycogen

Time	100 mg. takadiastase		10 cc. ultrafiltered fraction		200 mg takadias- tase, 100 mg. galactose, 100 mg. dextrose		10 cc ultrafiltered fraction, 100 mg. galactose, 100 mg dextrose	
	Mg. reducing substances found							
	Total	Net	Total	Net	Total	Net	Total	Net
<i>hrs</i>								
0	34	0	4	0	242	0	168	0
1.5	40	6	21.5	17 5	226	-16	135	-33
3	50	16	30	26	228	-14	160	-8
6	62	28	39	35	228	-14	160	-8
12	68	34	46 5	42 5	228	-14	168	0
24	85	51	55	51	242	0	170	2

TABLE XII
Influence of Various Sugars on Crude and Ultrafiltered Takadiastase in Absence of Glycogen

Time	100 mg takadiastase, 100 mg galactose, 100 mg. dextrose		10 cc ultrafiltered fraction, 100 mg galactose, 100 mg dextrose		50 mg. takadiastase, 50 mg. galactose		5 cc ultrafiltered fraction, 50 mg galactose		50 mg takadiastase, 200 mg galactose		50 mg. takadiastase, 200 mg dextrose		100 mg takadiastase, 100 mg glycogen (control)	
	Mg reducing substances found													
	Total	Net	Total	Net	Total	Net	Total	Net	Total	Net	Total	Net	Total	Net
hrs.														
0	167	0	156	0	52.5	0	37.5	0	160	0	182	0	40	0
1.5	138	-29	121	-35	42.5	-10	31.5	-6						
3	150	-17	121	-35	44	-8.5	31	-6.5						
5									134	-26	182	0	62.5	22.5
6	167	0	121	-35	52.5	0	28.5	-9						
9									121	-39	183	1	69	29
12	148	-19	125	-31	44	-8.5	28	-9.5	130	-30	185	3	74	34
24	148	-19	125	-31	43	-9.5	28.5	-9	130	-30	183	1	91	51

dextrose, or levulose. Since the sugars added in this as in all other experiments were not anhydrous, the reducing power as recorded

in all the experiments was less than the theoretical. The effects upon hydrolysis are recorded in Table IX.

Experiment 5—This experiment is identical with Experiment 4, except that the effects of mixtures of sugars rather than of single sugars were studied. The amount added was 100 mg. of each sugar. The data and results are given in Table X.

Experiment 6—In this experiment, the influence on the formation of reducing substances by crude and by ultrafiltered enzyme was studied in the presence as well as in the absence of glycogen. 1 gm. of takadiastase was ultrafiltered as described in a former paragraph. The non-ultrafiltrable fraction was taken up in 100 cc. of distilled water. The final volume was 100 cc. in each case. In the experiments of Table XI, each flask contained 100 mg. of glycogen; those of Table XII, except for the control, contained none.

Conclusion

We are not yet in a position to discuss in detail the action of takadiastase on glycogen with reference to intermediate products. Preliminary experiments tend to show that the ultrafiltrable product of the action of takadiastase upon glycogen contains not only a monosaccharide, when equilibrium is reached, but also a more complex saccharide. Whether this polysaccharide is a trisaccharide with a reducing power equivalent to one-third of that of glucose, as is that isolated by Barbour (6), or maltose as Norris (11) and others (12) believe it to be, we are not yet in a position to say.

It is interesting to note that at the point of equilibrium in Experiment 1, Table VI, the final product of glycogen hydrolysis amounts to 52 per cent of the total glycogen. Norris (11), using pancreatic diastase, found it to be 51.2 per cent.

In the experiment in which crude takadiastase was employed for the hydrolysis of glycogen (Experiment 2, Table VII), the mixture at the end of the experiment remained slightly opalescent while the opalescence of the mixture of the purified takadiastase and glycogen at a similar stage remained more pronounced. Most noteworthy in this connection was the finding that whenever to a mixture of glycogen and takadiastase, as in Experiments 4 and 5, fructose was added, the disappearance of opalescence was not only

rapid but complete before the end of the experiment. This phenomenon cannot be adequately interpreted at present, but it is possible that the keto group in fructose may be responsible. The other sugars investigated had no influence whatever on the disappearance of opalescence.

The addition of fructose does not seem to have any influence on the rate of hydrolysis of glycogen with takadiastase. This becomes quite obvious when the results of Experiment 4 are examined. The net amounts of reducing sugar that appeared at various intervals of time in the presence of levulose are almost identical with the amounts found when only glycogen and takadiastase were used.

The addition of dextrose on the other hand not only retards the rate of hydrolysis, but causes the amount of reducing sugar at the end of the experiment to be considerably less than in the control experiment. Thus, the net amount of reducing sugar found at the end of the experiment in the control containing glycogen and takadiastase is 52.5 mg. as compared with the net amount of 33 mg. where dextrose was added to a similar mixture, Experiment 4, Table IX. This experiment was checked over and over again with similar results. Experiments with takadiastase and dextrose alone, Table XII, show that there is no change in the amount of dextrose present beyond the experimental error.

The addition of galactose to a mixture of glycogen and takadiastase, as in Experiment 4, appears to influence the rate of reaction between glycogen and the enzyme. The final net amount of reducing sugar in the control experiment is 52.5 mg. as compared with 34 mg. when galactose was added to a similar mixture. On the other hand, in Experiment 6, Table XII, where 50 mg. of takadiastase and 200 mg. of galactose were used, there appeared a considerable drop at the end of the 24 hours. The drop from 160 to 130 mg. lies beyond the experimental error. This difference of 30 mg. cannot as yet be accounted for. On this basis no definite conclusion can be drawn as to whether or not the addition of galactose really influences the rate of hydrolysis of glycogen with takadiastase.

When dextrose and galactose were added together in equal quantities to takadiastase and glycogen as in Experiment 5, apparently there is complete inhibition of the action of the enzyme on glyco-

gen. This inhibition does not imply, however, that the intermediate products were not formed, but merely that the final reducing sugar was not. One observes further that at the end of the 7th hour when samples were removed, Experiment 5, for sugar determination, the net amount of reducing sugar is considerably less than at zero time. Similar results were obtained in Experiment 6, Table XI, except that the maximum drop in the net reducing sugar occurs at 1.5 hours instead of at the 7th hour. In Experiment 6, Table XII, where takadiastase and dextrose and galactose were added together a drop in the net reducing sugars is also noticeable. This finding necessitates a reconsideration of the idea that when dextrose and galactose are added together to a mixture of glycogen and takadiastase they inhibit the action of the enzyme. Seeking an interpretation one can therefore assume tentatively that a synthesis of a polysaccharide from dextrose and galactose might have occurred which might have either retarded the action of the enzyme or inhibited it. Further, the amount synthesized might have been equal to the amount hydrolyzed from glycogen, so that the total amount of reducing sugars present remained approximately constant throughout the experiment. Further investigations of this problem are in progress.

SUMMARY

The rate of hydrolysis of glycogen with either sulfuric or hydrochloric acid follows that of a pseudomonomolecular reaction. The specific rotatory power of the ultimate product is identical with that of glucose which is strong evidence that it is glucose. Phosphoric acid has a slower action on glycogen owing to its low dissociation constants.

The first change that occurs during the hydrolysis of glycogen with acids is the disappearance of opalescence. It is believed that the particle of glycogen is first reduced in size so that a non-opalescent glycogen is formed. This non-opalescent glycogen appears to have all the other characteristics of glycogen so far investigated and to give rise, when acted upon by acids, to an ultrafiltrable saccharide or mixture of saccharides which is the direct source of glucose.

The action of takadiastase on glycogen was studied. Apparently it causes the appearance of 52 per cent of the glycogen as

reducing sugars when equilibrium is reached. Whether a reducing sugar other than glucose is formed cannot be stated definitely. An ultrafiltrable polysaccharide is, however, formed. The addition of fructose to a mixture of takadiastase and glycogen causes the rapid disappearance of opalescence. Fructose does not influence in the least the rate of reaction between glycogen and this enzyme.

The addition of glucose on the other hand to a mixture of glycogen and takadiastase in solution alters to a considerable extent the rate of hydrolysis so that the amount of reducing sugars formed is less than when glucose is not present.

Galactose appears to have the same influence as glucose on a mixture of glycogen and takadiastase in solution. There is a possibility, however, that a synthesis of a polysaccharide may occur when takadiastase is allowed to act on a solution of glucose and galactose. In support of this view is the observation that there is a decrease in the amount of reducing sugars when takadiastase is allowed to act on a mixture of glucose and galactose alone.

A commercial preparation of takadiastase can easily be purified by ultrafiltration through a cellophane membrane. Most of the inorganic and organic impurities as well as the coloring matter pass through the membrane into the ultrafiltrates. The active enzyme is retained in the non-ultrafiltrable fraction.

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SOLUBILITY OF BONE IN SOLUTIONS OF MAGNESIUM SALTS

By J. C. FORBES

*(From the Department of Biochemistry, Medical College of Virginia,
Richmond)*

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In recent years a number of investigators have shown that the administration of magnesium salts increases calcium excretion. Injections of magnesium salts, especially intravenous injections, have given more positive evidence of calcium loss than oral administration. This is of course to be expected, since injection of the salt would naturally lead to a greater concentration of magnesium in the blood stream than would be obtained by oral administration.

Mendel and Benedict (1), working with dogs, cats, and rabbits, noted an increased calcium elimination in the urine when aqueous solutions of magnesium salts were injected subcutaneously. Schiff (2), studying the effect of magnesium sulfate on infants, found that the subcutaneous administration of 0.2 gm. of magnesium sulfate per kilo of body weight greatly increased the calcium excretion in the urine and slightly increased that in the feces. Stransky (3) studied the calcium and magnesium content of the blood of rabbits after the subcutaneous administration of sufficient magnesium sulfate to produce narcosis, and found that the plasma magnesium was greatly increased but the calcium concentration was definitely diminished. Richter-Quitner (4) corroborated this work and also found that, although the total amount of calcium in the blood was decreased, the amount of diffusible calcium was definitely increased.

The results following oral ingestion of magnesium salts have been more variable than those obtained by injection of the salt. Malcolm (5) produced a small increase in calcium excretion in dogs by feeding magnesium chloride. Hart and Steenbock (6) fed magnesium chloride and sulfate to swine and demonstrated an in-

creased calcium elimination in the urine but not in the feces. They also found that if soluble phosphate salts were fed with the magnesium salt, the calcium loss was considerably reduced. Palmer, Eckles, and Schutte (7) obtained similar results with cattle. Underhill, Honeij, and Bogert (8) found no material increase in calcium elimination in normal individuals, or in cases of leprosy, or of exostosis when the magnesium in the diet was increased. Bogert and McKittrick (9), however, obtained a slight increase in the urinary and fecal calcium of women to whom 6 gm. of magnesium lactate were given daily.

Further evidence showing the antagonistic action of magnesium on calcium utilization is given by the experiments of Shipley and Holt (10), and of Shelling, Kramer, and Orent (11), on the calcification of rachitic cartilage *in vitro*. These experiments show that magnesium has a specific inhibitory action on the bone-forming elements in rachitic metaphyses and this inhibitory action may be overcome by increasing the phosphate concentration of the solution. The experimental results of Holt, La Mer, and Chown (12), and of Zorkendorfer (13), further emphasize this antagonistic action of magnesium. They have shown that the solubility of poorly soluble calcium salts, such as $\text{Ca}_3(\text{PO}_4)_2$ and CaHPO_4 , in water, is greatly increased by the addition of soluble magnesium salts.

Although these results of Holt, La Mer, and Chown, and of Zorkendorfer, as well as the experiments on the injection of magnesium salts might lead one to assume that magnesium salts would increase the solubility of bone, no experimental evidence is available to show that this is the case. This investigation was therefore carried out in order to determine the effect of magnesium salts on the solubility of bone calcium and phosphorus *in vitro*, in the hope that further light might be thrown on the influence of magnesium salts on calcium metabolism.

Methods of Analyses—Calcium determinations were made by Kramer and Tisdall's (14) method, except that 50 cc. conical centrifuge tubes were employed, and as large a volume of solution as feasible was taken for analysis. Since magnesium interferes with calcium precipitation, as shown by Zorkendorfer (13), all of the solutions containing more than 0.004 M magnesium salt were diluted with water before precipitating the calcium. The

final magnesium concentration after addition of the ammonium oxalate solution was always less than 30 mg. per 100 cc. of solution. Phosphorus was determined by Fiske and Subbarow's (15) method. A quinhydrone hydrogen ion apparatus, unless otherwise stated, was used in all pH determinations.

TABLE I

Effect of Magnesium Salts on Solubility of Bone Calcium

Calcium values are expressed in mg. per 100 cc. of solution.

Experiment No.	Time of extraction	Ca concentration				Remarks
		0 M salt	0.008 M salt	0.08 M salt	0.4 M salt	
1	<i>hrs</i>					
	6	0.1	1.2	2.7	3.1	Bone in MgSO ₄ solutions in distilled water
	26	0.5	1.7	6.1	10.9	
	77	1.1	4.2	7.9	17.6	
	105	1.6	4.6	11.1	19.8	
2	6	1.0	2.1	4.7	8.6	Bone in MgSO ₄ solutions in m/15 phosphate buffer solution of pH 6.2
	26	1.1	2.5	8.1	20.2	
	77	1.9	3.7	13.9	35.0	
	105	1.2	2.6	19.6	52.8	
3	6	1.1	1.1	3.3	8.6	Bone in MgCl ₂ solutions in m/15 phosphate buffer solution of pH 6.2
	26	1.9	3.1	8.6	21.6	
	77	2.0	3.0	14.5	45.5	
	105	1.2	2.4	20.4	66.0	
4	6	2.9	4.6	8.5	25.4	Bone in MgSO ₄ solutions in m/15 KH ₂ PO ₄
	26	3.4	5.0	10.8	31.5	
	77	3.9	6.5	15.6	51.2	
	105	3.4	5.9	17.9	66.8	
5	6	0	0.1	0.3	2.3	Bone in MgSO ₄ solutions in m/15 phosphate buffer solution of pH 7.4
	26	0.4	0.8	3.7	11.6	
	77	0.5	0.7	5.7	22.0	
	105	0.3	0.6	6.2	29.0	

As preliminary experiments, the effect of various concentrations of magnesium sulfate and chloride on the solubility of bone calcium was studied, in the first case in m/15 phosphate buffer solutions of various hydrogen ion concentrations, and then in

TABLE II

Effect of Magnesium Sulfate on the Solubility of Bone Calcium in Ringer's Solution

Experiment No.	Time of extraction	pH of Ringer's solution	Ca concentration			Increase in Ca		
			0 M MgSO ₄	0.004 M MgSO ₄	0.04 M MgSO ₄	0 M MgSO ₄	0.004 M MgSO ₄	0.04 M MgSO ₄
	hrs.		mg.	mg.	mg.	mg.	mg.	mg.
1	0	7.8	13.6	13.6	13.6			
	16		7.4	11.6	24.1	-6.2	-2.0	+10.5
	24		7.7	11.7	22.6	-5.9	-1.9	+9.0
	24		7.5	11.5	21.7	-6.1	-2.1	+8.1
	24		6.7	12.9	21.8	-6.9	-0.7	+8.2
	48		9.2		23.1	-4.4		+9.5
	24		8.1	12.2	27.2	-5.5	-1.4	+13.6
2	0	7.2	11.9	11.9	11.2			
	26		5.8	9.1	20.7	-6.1	-2.8	+9.5
	24		5.1	9.8	19.5	-6.8	-2.1	+8.3
	48		6.3	13.2	18.3	-5.6	+1.3	+7.1
	24		7.8	11.7	18.3	-4.1	-0.2	+7.1
	48		6.1	9.6	18.1	-5.8	-2.3	+6.9
3	0	6.6	10.6	10.6	10.6			
	24		6.9	10.9	22.5	-3.7	+0.3	+11.9
	24			10.1	18.9		-0.5	+8.3
	48		6.6	11.1	19.1	-4.0	+0.5	+8.5
	24		7.3	11.8	18.0	-3.3	+1.2	+7.4
	48		6.5	10.8	20.0	-4.1	+0.2	+9.4
4	0	7.9	5.0	5.0	5.0			
	24		4.0	5.5	20.2	-1.0	+0.5	+15.2
	24		3.7	5.6	21.5	-1.3	+0.6	+16.5
	24		4.3	6.2	22.1	-0.7	+1.2	+17.1
	24		4.0	6.1	21.8	-1.0	+1.1	+16.8
5	0	6.7	5.0	5.0	5.0			
	24		4.8	7.1	21.4	-0.2	+2.1	+16.4
	24		3.5	7.0	21.8	-1.5	+2.0	+16.8
	24		4.0	7.8	20.8	-1.0	+2.8	+15.8

Ringer's solution containing various amounts of calcium chloride, and of different initial hydrogen ion concentrations. Beef rib bone was used in all experiments. It was first finely chopped, then washed, first with tap water and afterwards with distilled

water, until the supernatant fluid showed no visible signs of blood, and finally air-dried at room temperature.

In the experiments recorded in Table I approximately 10 gm. of this air-dried material were placed in 300 cc. Pyrex flasks and 200 cc. of the various buffer solutions added. Small amounts of

TABLE III
Solubility of Bone Calcium and Phosphorus in 0.04 M Magnesium Sulfate Solution in Distilled Water

Deter- mina- tion No.	Concentration per 100 cc.						Aver- age Ca:P ratio	Remarks
	Solution 1		Solution 2		Solution 3			
	Ca	P	Ca	P	Ca	P		
	mg.	mg.	mg.	mg.	mg.	mg.		
1	6.2	1.03	7.5	1.13	7.1	1.13	6.0	Solutions shaken for 1 hr. then stood 18 hrs. at room temperature
2	9.0	1.37	9.3	1.37	8.7	1.30	6.7	Additional $\frac{1}{2}$ hr. shaking and 18 hrs. standing at room temperature
3	2.8	0.73	2.8	0.64	2.2	0.62	3.9	Fresh solution added and shaken for 15 min. at room temperature
4	5.2	1.02	4.7	0.90	4.6	0.95	5.0	Additional 1 hr. shaking and 18 hrs. standing at room temperature
5	7.8	1.45	8.2	1.46	7.7	1.15	5.8	1 hr. additional shaking and 18 hrs. standing at room temperature
6	6.1	1.12	5.4	1.20	5.8	1.06	5.1	Fresh solution, left for 5 days at room tempera- ture
7	46.2	11.6					4.0	0.4 M MgSO ₄ solution added to large amount of bone and left in incu- bator for 3 days at 37°

chloroform and toluene were then added as preservatives, the flasks stoppered, and set aside at room temperature with occasional shaking. Samples for analysis were removed at intervals, filtered, and analyzed for calcium. The experimental results show that both magnesium sulfate and chloride exert a very great

solvent action on bone calcium, and that this action increases with increase in magnesium concentration.

In Table II is shown the effect of magnesium sulfate on the solubility of bone calcium in Ringer's solution containing various amounts of calcium. In order to obtain a maximum effect in a

TABLE IV

Effect of Magnesium Sulfate on Solubility of Bone Calcium and Phosphorus in Calcium Phosphate Solutions

Calcium and phosphorus values are expressed in mg. per 100 cc. of solution.

Experiment No	Analysis	Molar concentration of $MgSO_4$						Remarks
		0	0	0.004	0.004	0.04	0.04	
		mg	mg.	mg	mg.	mg.	mg	
1	Ca	9.4	9.4	9.4	9.4	9.4	9.4	Original solution
	P	2.9	2.9	2.9	2.9	2.9	2.9	
	pH	5.5	5.5	5.4	5.4	5.2	5.2	
2	Ca	1.1	1.7	2.1	2.4		23.4	Bone in solution for 24 hours
	P	1.0	1.2	1.7	1.3		1.4	
3	Ca	0.7	1.0		2.9	20.5	20.5	Bone in fresh solution for 24 hours
	P	0.9	1.3	2.1	1.2	1.5	1.5	
4	Ca	2.9	1.0	3.4	3.4	21.6	21.2	Bone in fresh solution for 72 hours
	P	0.8	1.0	1.0	0.9	1.3	1.4	
	pH	7.5	7.5	7.4	7.5	7.2	7.1	
5	Ca	1.8	0.8	5.6	3.7	22.6	22.6	Bone in fresh solution for 48 hours
	P	0.5	0.7	0.6	0.6	1.2	1.4	
	pH	7.7	7.7	7.5	7.5	7.1	7.1	
6	Ca	3.8	3.7	10.0	10.7	22.8	22.0	Bone in fresh solution for 120 hours
	P	0.3	0.4	0.7	0.8	0.9	0.6	

short time, these experiments were carried out in test-tubes, and a large amount of bone was used in comparison with that of the solution. At the end of each extraction period, the solutions were drained off and fresh solutions added for the next extraction. The results show that, in the absence of magnesium salts, some of the original solution calcium is removed by the bone; the greater the

original calcium concentration, the larger the amount removed. Magnesium sulfate, even in low concentrations, greatly inhibits this calcium deposition, and in high concentrations actually causes marked solution of the bone calcium. The relative effect of low concentrations of the magnesium salt is naturally influenced by the original calcium concentration of the solution. For example, when the original calcium concentration is 5 mg. per 100 cc. of solution and the magnesium concentration 0.004 M, slight solution of the bone calcium takes place, but when the original calcium concentration is 11.9 or above deposition of calcium takes place in the same magnesium concentration.

In Table III is shown the effect of magnesium sulfate on the solubility of bone calcium and phosphorus in distilled water. It will be seen that in all of the experiments the Ca:P ratio is considerably greater than the theoretical value for $\text{Ca}_3(\text{PO}_4)_2$. This is especially true in Determinations 1 and 2. Colorimetric determinations of hydrogen ions were made on the solutions in Determinations 2, 5, and 6 and were found to be approximately pH 7.8, 7.6, and 7.6 respectively, showing that the bone markedly decreased the hydrogen ion concentration of the solutions. It therefore seems probable that the bone calcium may have gone into solution in two ways, as a basic compound like $\text{Ca}(\text{OH})_2$ or CaCO_3 and as $\text{Ca}_3(\text{PO}_4)_2$.

In Table IV is shown the effect of magnesium sulfate on the solubility of bone calcium and phosphorus in a weak calcium phosphate solution. This solution was prepared by adding a small amount of calcium chloride to a weak solution of monopotassium phosphate. As in the previous experiment, the bone caused a marked decrease in the hydrogen ion concentration of the solutions, both in the absence and presence of magnesium salt. Phosphorus concentration was also decreased in all solutions. The calcium concentration in the solutions containing no magnesium salt and those containing 0.004 M magnesium sulfate was decreased, but that of the solutions containing 0.04 M magnesium sulfate was markedly increased. The decrease in calcium and phosphorus, especially in the absence of magnesium salts, is likely due to a considerable extent to the conversion of the soluble $\text{Ca}(\text{H}_2\text{PO}_4)_2$ into the more insoluble CaHPO_4 and $\text{Ca}_3(\text{PO}_4)_2$ due to the decreased acidity of the solution.

TABLE V

Effect of Magnesium Sulfate on Solubility of Bone Calcium and Phosphorus in Weak Phosphate Buffer Solutions

Calcium and phosphorus values are expressed in mg. per 100 cc. of solution.

Determination No	Molar concentration of $MgSO_4$									Remarks
	0			0.004			0.04			
	Ca	P	pH	Ca	P	pH	Ca	P	pH	
1	0	6.6	6.20	6.6	6.0	0	6.5	5.9	Original solution	
2	0.12	2.4	7.60	1.1	7.5	11.3	1.6	7.4	Solution in contact with bone for 75 min. at 37°	
3	0.24	5.4	7.61	1.6	2.4	7.5	9.3	3.0	7.2	Fresh solution, in contact with bone for 75 min. at 37°
4		5.4	7.62	2.4	3.0	7.4	9.4	4.6	7.1	Fresh solution, in contact with bone for 90 min. at 37°
5	0.6	5.0	7.42	2.4	1.9	7.2	9.8	3.5	7.0	Fresh solution, in contact with bone for 3 hrs. at 37°
6	0.54	4.4	7.42	2.4	2.0	7.3	7.6	3.6	7.2	Fresh solution, in contact with bone for 5 hrs. at 37°
7	0.7	3.4	7.72	2.3	1.8	7.4	12.4	2.2	7.3	Fresh solution, in contact with bone for 28 hrs. at 37°
8	0.46		7.22	2.3	3.5	7.0	9.9	4.9	6.9	Fresh solution, in contact with bone for 3 hrs. at 37°
9	0.5	5.2	7.42	2.6	3.6	7.0	7.9	5.1	7.2	Fresh solution, in contact with bone for 6 hrs. at 37°
10	0.24	4.7	7.62	2.2	2.8	7.6	8.8	4.0	7.2	Fresh solution, in contact with bone for 15 hrs. at 37°
11	1.0	5.9	6.92	2.5	3.6	6.9	5.7	5.7	6.7	Fresh solution, in contact with bone for 150 min. at 37°
12	0.6	5.1	7.02	2.3	4.6	7.0	6.6	6.2	6.9	Fresh solution, in contact with bone for 15 hrs. at 37°

25 cc. of solution were used for each extraction except in Determinations 11 and 12 where 75 cc. were used. Solutions were shaken occasionally during the extraction.

In Tables V and VI are presented experimental data showing the effect of magnesium sulfate on the solubility of bone in weak phosphate buffer solutions. The results are in agreement with

TABLE VI

Effect of Magnesium Sulfate on the Solubility of Bone Calcium and Phosphorus in Weak Phosphate Buffer Solutions

Calcium and phosphorus values are expressed in mg. per 100 cc. of solution.

Experiment No.	Analysis	Concentration of Mg salt					Remarks
		0 M	0.0016 M	0.004 M	0.04 M	0.2 M	
1	Ca	0	0	0	0	0	Original solution
	P	6.6	6.6	6.6	6.6	6.6	
	pH	6.1	6.1	6.0	5.8	5.3	
2	Ca	1.3	2.3	3.1	13.4	23.5	10 gm. bone added to 25 cc. respective solution and left 2 hrs. at 37°
	P	4.6	4.2	4.2	4.6	7.2	
	pH	7.3	7.1	7.0	6.9	6.1	
3	Ca	1.1	1.4	2.4	9.8	12.1	Fresh solution added and left 2 hrs. at 37°
	P	6.2	5.8	5.9	6.2	8.8	
	pH	7.0	7.0	6.7	6.5	6.4	
4	Ca	0.6	1.2	2.8	8.5	13.0	Fresh solution added and left 3 hrs. at 37°
	P	5.4	5.5	5.4	6.0	8.8	
	pH	7.0	6.9	6.6	6.6	6.4	
5	Ca	0.4	0.4	2.1	10.3	18.2	Fresh solution added and left 18 hrs. at 37°
	P	4.8	5.0	3.9	4.6	7.3	
	pH	7.5	7.3	7.3	6.8	6.5	
6	Ca	0.7	1.1	2.8	7.4	14.2	75 cc. fresh solution added and left 24 hrs. at 37°
	P	5.7	5.9	6.4	6.6	8.7	
	pH	6.9	6.9	6.8	6.7	6.6	
7	Ca	0.9	1.3	3.2	8.3	15.7	75 cc. fresh solution added and left 24 hrs. at 37°
	P	5.7	5.8	6.6	6.6	8.8	
	pH	7.1	7.0	6.9	6.9	6.9	

those obtained in the previous experiments. The decrease in acidity was very marked in all cases. The rapidity of this decrease will of course depend on various factors, such as the size of

the bone particles and the relative amounts of bone and solution. In an unrecorded experiment it was found that when only sufficient solution to cover the bone was used, 10 minutes at 37° sufficed to raise the pH of a M/450 phosphate buffer solution from 6.2 to 7.2. The speed with which this change is brought about, as shown by Table V, decreases with increased extraction. This is, of course, to be expected since a certain amount of the basic compounds of the bone are removed with each extraction. The calcium solubility, as in the previous experiments, increased with increase in magnesium concentration. The phosphate concentration was

TABLE VII

Solubility of Bone Calcium and Phosphorus in 0.022 M Magnesium Lactate in M/450 Phosphate Buffer Solution of pH 5.9

Deter- mination No	Concentration per 100 cc.						Remarks
	Solution 1		Solution 2		Solution 3		
	Ca	P	Ca	P	Ca	P	
	mg	mg.	mg.	mg	mg.	mg	
1	0	6.8	0	6.8	0	6 8	Original solution
2	2 1	7 3	2 1	7.3	2 6	7 2	2 hrs. shaking at room tem- perature
3	4 7	6.8	4 2	6.7	3 8	6 7	1½ hrs. additional shaking
4	3 6	7 0	3 8	6 8	4 4	6 5	Fresh solution, shaken 2 hrs. then stood 18 hrs. at room temperature
5	5 0	6 9	5.1	6.6	5 7	6.5	1½ hrs. additional shaking then 18 hrs. standing at room temperature

diminished in all solutions except the 0.2 M magnesium sulfate solutions in which a slight increase was obtained. In several unrecorded experiments it has been found that, even in this concentration, a definite decrease in the phosphate concentration of the solution is obtained, if the extraction period is sufficiently prolonged.

A somewhat similar experiment showing the effect of magnesium lactate on the solubility of bone is shown in Table VII. It will be seen that the lactate has the same general effect on the bone calcium and phosphorus as the other magnesium salts. The experi-

ments were not sufficiently prolonged to show the maximum calcium solubility, but they show definitely a great increase in calcium solubility over that obtained in similar buffer solutions in the absence of magnesium salts, as shown in Tables I to VI and VIII. The decrease in phosphorus after the preliminary rise was not as marked as in the other experiments. This, however, is likely primarily due to the short extraction periods.

TABLE VIII

Effect of Phosphate Concentration on Solubility of Bone Calcium in 0.004 M Magnesium Sulfate

Calcium values are expressed in mg. per 100 cc. of solution; pH of solutions 6.2.

Experiment No.	Phosphate concentration				Remarks
	0 M	M/750	M/150	M/15	
1	2.4	2.2	1.9	1.6	6 hrs. extraction with occasional shaking at 37°
	3.1	2.9	1.7	1.3	Additional 24 hrs. at 37°
	2.3	2.1	1.1	1.0	" 24 " " 37°
	2.8	2.1	1.4	1.1	" 24 " " 37°
2	3.6	3.8	2.6		6 hrs. extraction with occasional shaking at 37°
	4.9	5.0	3.0		Additional 24 hrs. at 37°
	5.0	4.6	2.9		" 24 " " 37°
	4.9	4.0	2.6		" 24 " " 37°
3	2.9	2.2	1.9	1.6	17 hrs extraction with occasional shaking at 37°
	2.7	2.2	1.9	1.6	Additional 24 hrs. at 37°
	2.5	2.0	1.4	1.1	" 24 " " 37°

The bone used in Experiment 2 was heated in an oven at 100° for several days. This may explain the greater solubility.

Since an increase in phosphate concentration decreases the antagonistic action of magnesium both on calcification *in vitro* and calcium retention in experimental animals, it was decided to study the effect of variations in phosphate concentrations on the solubility of bone calcium in magnesium solutions. The experimental data are recorded in Table VIII. It will be seen that the

phosphate concentration has a slight but very definite effect; *i.e.*, the greater the phosphate concentration the less the calcium solubility.

DISCUSSION

The experimental results show that soluble magnesium salts greatly increase the solubility of bone calcium in water solutions; the greater the magnesium concentration the greater the calcium solubility. The effect of the magnesium salts is, however, more or less influenced by the calcium and phosphate concentration of the original solution. It will be seen, for example, in Table III that magnesium sulfate in distilled water causes bone calcium to go into solution probably as a phosphate and as some other calcium compound having basic properties, possibly the hydroxide or carbonate. That such is likely the case is evident from the fact that the Ca:P ratio of the solutions is in all cases at least double that of the theoretical value for $\text{Ca}_3(\text{PO}_4)_2$, and also from the fact that the bone markedly decreased the hydrogen ion concentration of the solutions. If, on the other hand, calcium is present in the original solution, either solution of the bone calcium or deposition of the solution calcium on the bone may take place, depending on the calcium, phosphate, and magnesium concentration of the solution. For example, from the results recorded in Table II, it will be seen that in the 0.004 M magnesium solutions, deposition of calcium took place when the original calcium concentration was 11.9 mg. per 100 cc., or over, but solution of bone calcium took place when it was only 5 mg. per 100 cc. At the higher magnesium concentrations, solution of bone calcium took place at all calcium concentrations employed. In a similar manner, variations in the phosphate concentration of the solution influence the effect of magnesium on calcium solubility, especially in solutions of low magnesium concentrations. This effect of phosphates may be seen by comparing the results obtained with the 0.004 M magnesium sulfate in Ringer's solution with those obtained with a weak phosphate solution containing the same amount of magnesium and approximately the same calcium concentration. For example, in Experiment 3, Table II, there is a slight but definite increase in the solubility of the bone calcium in the 0.004 M magnesium sulfate solution over that in the Ringer's solution

itself. On the other hand, as shown in Table IV, in solutions containing the same magnesium concentration and approximately the same calcium concentration but containing 2.9 mg. of phosphorus per 100 cc., definite deposition of solution calcium took place. Further evidence showing the effect of phosphates on the calcium solution action of magnesium salts is given in Table VIII. It will be seen from Table VIII that although there is no very definite difference between the calcium solubility in distilled water and the M/750 phosphate buffer solution (4.1 mg. of phosphorus per 100 cc.), there is a very marked difference when one compares the calcium solubility in the solutions of higher phosphate concentrations with that in distilled water containing the same amount of magnesium salt.

Since an increase in either the calcium or phosphate concentration of the solution decreases the solution effect of magnesium salts, it is probable that the magnesium acts by increasing the solubility product constant of the calcium salts rather than by the formation of complex calcium-magnesium salts as suggested by Zorkendorfer (13). This would also explain how, as shown in many experiments, marked solution of bone calcium may take place with an actual decrease in the phosphate concentration of the solution. It is probable that this decrease is due to precipitation of $\text{Ca}_3(\text{PO}_4)_2$; consequently the calcium and phosphate concentrations at equilibrium are determined by the solubility product constant of this compound under the conditions of the experiment. The fact that the bone calcium passes into solution in some other form beside $\text{Ca}_3(\text{PO}_4)_2$ makes it possible to obtain marked solution of bone calcium in spite of an actual decrease in the phosphate concentration of the solution.

SUMMARY

The solubility of bone in various solutions of magnesium salts has been studied and it has been found that:

1. The solubility of bone calcium in water solutions is greatly increased by magnesium salts; the greater the magnesium concentration the greater the calcium solubility.
2. In the absence of phosphates from the original solution the calcium goes into solution as a phosphate and as some other compound, possibly a basic compound like the hydroxide or carbonate.

3. When phosphates are present to even a moderate extent in the original solution, marked solution of bone calcium may take place with an actual decrease in the phosphate concentration of the solution.

4. Increasing the calcium or phosphate concentration of the original solution decreases the solution action of magnesium salts, especially when these are present in low concentrations.

5. Bone has been found to have a marked neutralizing action against acids, the pH of all weakly acid solutions being rapidly increased to over 7 by the neutralizing action of the bone.

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STUDIES ON THE PHYSIOLOGY OF PYRIMIDINES

IV. FURTHER EXPERIMENTS ON THE INTERMEDIARY METABOLISM OF URACIL*

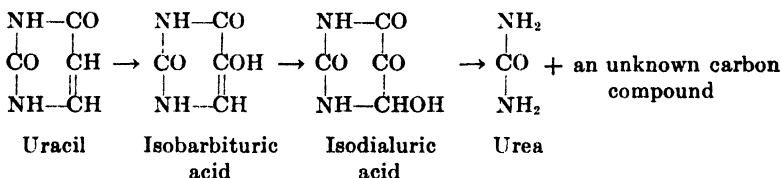
BY LEOPOLD R. CERECEDO

WITH THE TECHNICAL ASSISTANCE OF ETHEL F. CURRY, JAKOB A. STEKOL,
AND HENRY ELBAUM

(From the Division of Biochemistry, University of California Medical School,
Berkeley)

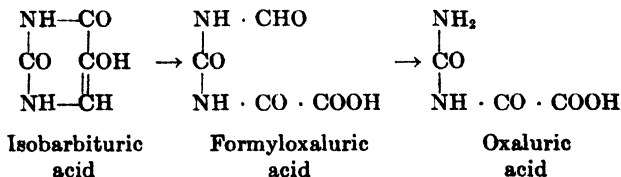
(Received for publication, June 22, 1931)

Experiments reported in a previous paper (1) led the writer to assume that in the metabolism of uracil in the dog we are dealing with the following sequence of reactions.



The present investigation was undertaken with the view of finding the oxidation product of isodialuric acid, which gives rise to urea in the degradation of uracil in the dog.

It has been shown by Offe (2) that formyloxaluric acid and oxaluric acid are intermediate products in the oxidation of isobarbituric acid *in vitro* by permanganate. We are dealing with the following reaction.



* This investigation has been aided by a grant from the National Research Council.

Quite recently, Johnson and Flint (3) have found that formyloxaluric acid and oxaluric acid are also products of the oxidation of uracil by ozone.

We attach great significance to the findings of Offe and of Johnson and Flint for the following reasons. The experiments of Biltz and Schauder (4) have shown that oxaluric acid is formed in the oxidation of uric acid by permanganate in a weak acid medium. The same substance may also be obtained as end-product of the oxidation of uric acid glycol, alloxan, and allantoin by hydrogen peroxide.

These findings on the oxidation of these substances *in vitro*, leading to the same end-product, namely oxaluric acid, suggested the idea that this compound might be a link connecting the catabolic breakdown of purines and pyrimidines in the animal body. This conception gains some support from the fact that oxaluric acid is known to be present in small amounts in the urine (5).

If it could be shown that oxaluric acid is metabolized in the animal body to yield urea, this evidence, in addition to the findings of Biltz and Schauder, and other workers, on the oxidation of uric acid *in vitro*, would favor the view that oxaluric acid is the precursor of the urea found in increased amounts in the urine after the ingestion of certain purine compounds, as reported by Schittenhelm and Wiener (6) and by Severin (7).

The purpose of the present investigation was to study the fate of oxaluric acid and formyloxaluric acid in the dog. There is only one investigation on record dealing with the metabolism of oxaluric acid. Luzzatto (8) injected this substance subcutaneously into dogs and found that it was completely oxidized.

EXPERIMENTAL

Oxaluric acid was prepared according to the method of Biltz and Schauder (4). In the synthesis of formyloxaluric acid we followed the directions of von Gorski (9). The purity of the synthesized products was checked by Kjeldahl nitrogen determinations. Female dogs were used exclusively in the experiments described below. The animals were kept on the standard diet described by Cowgill (10). The plan of the experiments was to follow the various urinary constituents until a constant nitrogen output had been reached, to give the substance to be tested either mixed with

the food or by injection, then to continue the experiment until the output of these excretory products had returned to normal. The dogs were kept in metabolism cages, and the urine collected by catheterization every 24 hours. The animals were allowed to drink water *ad libitum*.

The following analytical methods were used: total N, Kjeldahl; urea, Van Slyke's gasometric method; ammonia, Folin's permutit method; inorganic sulfur, precipitation with benzidine and titration according to Drummond.

Experiments with Oxaluric Acid

The results obtained after feeding oxaluric acid are shown in Table I. An examination of the data reveals that the substance

TABLE I
Experiments with Oxaluric Acid

Dog 3; weight 10.8 kilos.

Day	Urine volume	Total N	Urea N	Ammonia N	Inorganic S
	cc	gm	gm.	gm	gm.
1	129	4.98	4 27	0.21	0 194
2	90	4 96	4 26	0.22	0 205
3	133	5.06	4 30	0.19	0 198*
4	116	5.73	4 45	0.33	0 216
5	97	4 93	4 17	0.26	0.209
6	103	4.91	4 21	0.25	0.205

* 2.5 gm. of oxaluric acid were fed with the food. N = 0.53 gm.

was to some extent catabolized to urea. Two experiments were conducted in which the oxaluric acid was injected subcutaneously in the form of its ammonium salt. The data are found in Table II. In both cases a rise in the urea output was noted after the injection of this substance.

In these two experiments we determined also the oxalic acid in the urine. We found in both cases a pronounced rise in the oxalic acid output, amounting to more than a 5-fold increase. The inaccuracy of the present day methods for the determination of oxalic acid in the urine did not justify the inclusion of these data in the tables.

Experiments with Formyloxaluric Acid

For the reasons stated in the introduction, it seemed desirable to investigate the fate of formyloxaluric acid, the precursor of oxaluric

TABLE II
Experiments with Oxaluric Acid

Day	Dog 7, weight 9.0 kilos					Dog 3, weight 10.8 kilos				
	Urine volume	Total N	Urea N	Ammonia N	Inorganic S	Volume	Total N	Urea N	Ammonia N	Inorganic S
	cc.	gm.	gm.	gm.	gm.	cc	gm	gm	gm.	gm.
1	120	4.91	4.12	0.38	0.303	106	4.98	4.26	0.39	0.265
2	117	4.78	4.05	0.36	0.306	90	4.80	4.13	0.34	0.273
3	95	4.66	3.90	0.38	0.299	108	4.89	4.23	0.36	0.262
4	125	4.79	3.99	0.38	0.310*	120	4.90	4.23	0.36	0.262†
5	145	5.60	4.28	0.55	0.310	191	5.79	4.59	0.50	0.259
6	95	4.61	3.84	0.38	0.295	110	5.00	4.26	0.45	0.256
7	101	4.86	4.06	0.41	0.313	125	4.75	4.00	0.33	0.259
8	115	4.83	4.05	0.40	0.313	140	4.78	4.12	0.32	0.276

* 2.4 gm. of ammonium oxalurate in 40 cc. of H₂O were injected subcutaneously. N = 0.68 gm.; urea N = 0.42 gm.; NH₃-N = 0.22 gm.

† 2.5 gm. of ammonium oxalurate in 40 cc. of H₂O were injected subcutaneously. N = 0.7 gm.; urea N = 0.47 gm.; NH₃-N = 0.23 gm.

TABLE III
Experiments with Formyloxaluric Acid

Day	Dog 3, weight 10.9 kilos					Dog 8, weight 10.7 kilos			
	Urine volume	Total N	Urea N	Ammonia N	Inorganic S	Volume	Total N	Urea N	Inorganic S
	cc.	gm	gm.	gm	gm	cc.	gm	gm.	g
1	125	5.30	4.40	0.53	0.388	120	4.38	3.30	0.
2	115	5.34	4.34	0.48	0.396	120	4.21	3.18	0.
3	130	5.40	4.41	0.52	0.390*	100	4.26	3.19	0.
4	185	5.90	4.60	0.66	0.420	115	4.56	3.44	0.
5	183	5.28	4.26	0.54	0.408	105	4.35	3.32	0.
6	145	5.43	4.34	0.58	0.403	100	4.22	3.21	0.
7	140	5.39	4.27	0.54	0.414				

* 3.0 gm. of formyloxaluric acid were fed with the food. N = 0.53 gm.

† 2.5 gm. of formyloxaluric acid were fed with the food. N = 0.44 gm.

acid in the oxidation of isobarbituric acid *in vitro*. The data from two experiments illustrating the catabolism of this compound are

shown in Table III. In both cases we find a distinct rise in the urea output after feeding formyloxaluric acid. Attempts to investigate the breakdown of this substance in the body, when injected, were unsuccessful. The compound was found to be very toxic.

DISCUSSION

We believe that the results obtained with oxaluric acid justify the assumption that this substance is an intermediary metabolite of uracil. There is evidence to show that it may play also a certain rôle in the degradation of purines in the animal body: in the first place, the finding of oxaluric acid as being one of the main products of the oxidation of uric acid by various reagents *in vitro*; secondly, the occurrence of oxaluric acid in small amounts in the urine; lastly, the increased urea output observed after the ingestion of certain purine bodies.

The fact that formyloxaluric acid was found to be toxic, when injected, does not preclude the possibility that it may be the progenitor of oxaluric acid. Perhaps the concentration of the substance in the injection fluid was too high. When fed by mouth, formyloxaluric acid has an effect on the neutral sulfur fraction of the urine which is very similar to that shown by isobarbituric acid. These findings will be the subject of a future publication.

SUMMARY

1. Experiments are described in which oxaluric acid and formyloxaluric acid were fed to dogs. Evidence was obtained that these compounds are partly catabolized to yield urea.

2. When injected subcutaneously, oxaluric acid is broken down in a similar manner as when fed by mouth. Formyloxaluric acid, on the other hand, when injected, was found to be toxic.

3. On the basis of these findings it is assumed that in the metabolism of uracil we are dealing with the following sequence of reactions: uracil \rightarrow isobarbituric acid \rightarrow isodialuric acid \rightarrow oxaluric acid \rightarrow urea + oxalic acid.

4. The significance of oxaluric acid as a possible connecting link in the catabolism of purines and pyrimidines is pointed out.

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STUDIES ON THE PHYSIOLOGY OF PYRIMIDINES

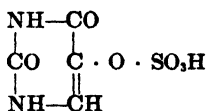
V. ON THE EFFECTS OF CERTAIN PYRIMIDINES ON THE SULFUR METABOLISM OF THE DOG*

By JAKOB A. STEKOL AND LEOPOLD R. CERECEDO

(From the Division of Biochemistry, University of California Medical School, Berkeley)

(Received for publication, June 29, 1931)

It was observed by Cerecedo (1) that after feeding isobarbituric acid to dogs there was a distinct decrease in the urinary output of inorganic sulfates and a corresponding rise in the ethereal sulfur fraction. These findings led him to assume tentatively that isobarbituric acid was partly excreted in the urine in conjugation with sulfuric acid, as an ethereal sulfate. The structure of this substance may be represented thus:



The present investigation was undertaken in an attempt to isolate the isobarbituric acid sulfate from the urine. Furthermore, it seemed desirable to make a more thorough study of the catabolism of isobarbituric acid in the dog.

EXPERIMENTAL

Isobarbituric acid was prepared according to the method of Davidson and Baudisch (2). For a description of the experimental technique we refer to the previous publication (1).

Experiments with Isobarbituric Acid

In Table I are shown the results obtained after feeding 2.5 gm. of isobarbituric acid. We find a rise in the urea output, a drop in

* This investigation has been aided by a grant from the Research Board of the University of California.

in the inorganic sulfur fraction, and a corresponding increase in the ethereal sulfates. Very striking is the effect of isobarbituric acid on the neutral sulfur. There is no detectable amount of neutral sulfur in the urine on the day after feeding the compound.

TABLE I

Experiments with Isobarbituric Acid

Dog 8; weight 10.0 kilos in first series; 10.7 kilos in second series.

Day	Urine volume	Total N	Urea N	Inorganic S	Total S	Total sulfate S	Ethereal S	Neutral S
	cc.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	190	3.52	2.76	0.230	0.275	0.241	0.011	0.034
2	95	3.59	2.83	0.227	0.266	0.242	0.015	0.024
3	101	3.80	2.89	0.243	0.304	0.263	0.020	0.041*
4	225	4.38	3.20	0.108	0.266	0.267	0.159	
5	145	3.76	2.93	0.238	0.284	0.269	0.031	0.015
6	250	3.73	2.96	0.238	0.274	0.258	0.020	0.016
7	75	3.73	2.93	0.238	0.255	0.255	0.017	0.019
8	137	3.81	2.98	0.248	0.263	0.263	0.015	0.028
9	144	3.70	2.97	0.226	0.249	0.249	0.023	0.035*
10	105	4.25	3.30	0.073	0.252	0.252	0.179	
11	111	3.76	3.02	0.245	0.279	0.279	0.034	0.028
12	91	3.84	3.08	0.246	0.268	0.268	0.022	0.021
1	80	3.82	2.92	0.265	0.326	0.291	0.026	0.035
2	105	3.98	2.99	0.263	0.322	0.287	0.024	0.035
3	85	3.93	2.94	0.261	0.313	0.281	0.020	0.027*
4	165	4.52	3.44	0.110	0.273	0.274	0.164	
5	105	4.24	3.17	0.283	0.353	0.319	0.036	0.034
6	95	4.06	3.02	0.271	0.327	0.297	0.026	0.030
7	115	3.88	2.90	0.270	0.314	0.288	0.018	0.026†
8	120	4.38	3.30	0.167	0.293	0.287	0.120	0.006
9	120	4.21	3.18	0.291	0.333	0.313	0.022	0.020
10	100	4.26	3.19	0.287	0.329	0.304	0.017	0.025

* 2.5 gm. of isobarbituric acid fed with food; N = 0.55 gm.

† 2.0 gm. of isobarbituric acid fed with food; N = 0.44 gm.

In order to ascertain whether a proportionately larger amount of the substance would be catabolized to urea, when fed in smaller quantities, we gave to the same dog 2.0 gm. of isobarbituric acid. An examination of the data recorded in the last four readings of Table I shows that after feeding the substance the neutral sulfur

does not completely disappear, as was observed after the ingestion of 2.5 gm. of isobarbituric acid.

In Table II we present the data obtained after feeding a still smaller amount of this compound. In this experiment the dog ingested 1.8 gm. of isobarbituric acid. The results obtained are similar to those observed after feeding 2.0 gm. of the substance.

If we calculate the rise in ethereal sulfates after feeding isobarbituric acid in each experiment, and assume for the sake of argu-

TABLE II
Experiments with Isobarbituric Acid

Dog 10; weight 10.0 kilos.

Day	Urine volume	Total N	Urea N	Inorganic S	Total S	Total sulfate S	Ethereal S	Neutral S
	cc.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	156	3.70	2.88	0.218	0.267	0.239	0.021	0.028
2	85	3.75	2.88	0.230	0.283	0.252	0.022	0.031
3	127	3.67	2.80	0.229	0.281	0.250	0.021	0.031
4	260	3.81	2.93	0.235	0.289	0.256	0.021	0.033*
5	165	4.26	3.16	0.120	0.258	0.253	0.133	0.005
6	90	3.96	3.00	0.240	0.292	0.263	0.023	0.029
7	127	3.90	2.96	0.237	0.290	0.258	0.021	0.032

* 1.8 gm. of isobarbituric acid fed with food; N = 0.39 gm.

ment that this increase is due to the presence of isobarbituric acid sulfate, we obtain the following data:

Isobarbituric acid fed	Excreted as isobarbituric acid sulfate
gm.	per cent
2.5	22
2.5	23
2.5	27
2.0	23
1.8	30

Attempts to Isolate Isobarbituric Acid Sulfate from the Urine

Fosse (3) has shown that compounds containing a CO group—
 NH—
 NH—

ing react with xanthidrol to form condensation products which

are very little soluble in water. It seemed of interest to test whether isobarbituric acid would also react with xanthidrol. Preliminary experiments were carried out with the object of recovering isobarbituric acid which had been added to urine. The procedure was as follows:

0.2 gm. of isobarbituric acid was dissolved in 75 to 80 cc. of dog urine. To this mixture an excess of basic lead acetate was added. After standing for several hours, the precipitate was filtered and washed thoroughly with cold water. The substance remaining on the filter was then suspended in cold water and treated with hydrogen sulfide. This removed the lead in the form of lead sulfide, which was filtered off. The filtrate was concentrated *in vacuo* at 30–40° to a small volume. The residue was treated with an equal volume of glacial acetic acid. On adding 0.4 gm. of xanthidrol in a volume of glacial acetic acid equal to that of the solution to be tested, a white precipitate fell out. The substance was filtered and washed with water. After drying, the compound was analyzed for nitrogen with the following results:

$C_4H_4O_6N_2$. Calculated, N 5.70; found, N 5.28

For the isolation of isobarbituric acid sulfate from the urine after feeding isobarbituric acid we proceeded as follows:

The 24 hour sample of urine collected after feeding the compound was treated with an excess of basic lead acetate and allowed to stand for 5 to 6 hours. After removing the lead in the form of lead sulfide, we tested the filtrate (I) for the presence of isobarbituric acid, since the results obtained in our feeding experiments had shown that the substance was partly excreted unchanged. On examining the solution for isobarbituric acid according to the method of Davidson and Baudisch (2), we found that it gave a positive reaction. On concentrating the filtrate (I), which was obtained after separation of the lead sulfide, to a small volume *in vacuo*, a brown precipitate fell out. This substance was filtered, washed, and dried. Qualitative tests showed that it contained sulfur. The compound was analyzed for nitrogen with the following results.

$C_4H_4O_6N_2S$ (isobarbituric acid sulfate). Calculated. N 13.46
Found. " 14.7

These data show that we were dealing with an impure preparation.

The filtrate (II) of the above substance was treated with an equal volume of glacial acetic acid. To this mixture a solution of 0.5 gm. of xanthydrol in glacial acetic acid was added. After allowing the mixture to stand for several hours, a white precipitate separated out. It was filtered, washed with water, and analyzed for nitrogen.

$C_{10}H_{10}O_6N_2S$ (dixanthdrylisobarbituric acid sulfate).

Calculated. N 4.93

Found. " 4.51

" 5.00

TABLE III

Experiments with Isodialuric Acid

Dog 8; weight 10.7 kilos.

Day	Urine volume	Total N	Urea N	Inorganic S	Total S	Total sulfate S	Ethereal S	Neutral S
	cc.	gm.	gm.	gm	gm.	gm.	gm.	gm.
1	108	4.28	3.40	0.288	0.343	0.305	0.017	0.038
2	110	4.30	3.48	0.290	0.345	0.306	0.016	0.039
3	100	4.24	3.41	0.289	0.339	0.305	0.016	0.034*
4	375	5.00	3.65	0.263	0.279	0.283	0.020	
5	220	4.10	3.25	0.299	0.330	0.317	0.018	0.013
6	165	4.24	3.41	0.304	0.346	0.304	0.016	0.026

* 3.0 gm. of isodialuric acid injected subcutaneously; N = 0.58 gm.

The brownish precipitate, which had separated from the concentrated filtrate (I) of the lead sulfide, was redissolved in water and treated with xanthydrol, as described above. A substance separated out on standing, which on analysis, gave the following figures.

$C_{10}H_{10}O_6N_2S$. Calculated, N 4.93; found, N 4.40

Experiments with Isodialuric Acid and Formyloxaluric Acid

The results observed in the above experiments made it desirable to study the fate of other possible breakdown products of uracil in the animal body. We chose for this purpose isodialuric acid and formyloxaluric acid. These compounds, when fed to dogs, have been shown to undergo a breakdown in metabolism similar to that of isobarbituric acid (1, 4).

Isodialuric acid was prepared according to the method of Behrend and Roosen (5). In the synthesis of formyloxaluric acid we followed the directions of von Gorski (6). The data obtained after injection 3.0 gm. of isodialuric acid are recorded in Table III. An examination of the figures shows that on the day after the injection of this substance there is no detectable amount of neutral sulfur present in the urine. In contrast with the observations made with isobarbituric acid, we find no increase in the ethereal sulfate fraction.

In Table IV we present the data obtained after feeding formyloxaluric acid. We find also in this case a pronounced drop in the neutral sulfur fraction of the urine.

TABLE IV
Experiments with Formyloxaluric Acid

Dog 10; weight 10.7 kilos.

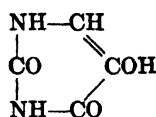
Day	Urine volume	Total N	Urea N	Inorganic S	Total S	Total sulfate S	Ethereal S	Neutral S
	cc	gm	gm	gm	gm.	gm	gm.	gm
1	120	4 21	3 18	0 291	0 333	0 313	0 022	0 020
2	100	4 26	3 19	0 287	0 329	0 304	0 017	0 025*
3	115	4 56	3 44	0 286	0 310	0 304	0 018	0 006
4	105	4 35	3 32	0 299	0 351	0 314	0 015	0 037
5	100	4 22	3 21	0 290	0 337	0 305	0 015	0 032

* 2.5 gm. of formyloxaluric acid fed with food; N = 0.33 gm.

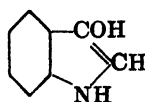
DISCUSSION

The observations reported in the present study seem to us worthy of notice for several reasons. In the first place, the results so far obtained in the investigation of the substance, which is excreted by dogs in the urine after the ingestion of isobarbituric acid, seem to indicate that we are dealing with an entirely new type of ethereal sulfates. Heretofore, the only substances which have been known to combine with sulfuric acid in the animal organism were phenol and indoxyl and their derivatives.

It is of interest in this connection to recall the experiments of Davidson and Baudisch (2) on the oxidation of isobarbituric acid. These workers have called attention to the similarity in structure of isobarbituric acid (I) and indoxyl (II).



I



II

The observations made on the effects of isobarbituric acid, isodialuric acid, and formyloxaluric acid on the sulfur metabolism of the dog are difficult of interpretation. An examination of Tables I and II reveals that the increase in ethereal sulfates corresponds to the drop in the inorganic sulfur fraction. Unexplainable at the present time is the disappearance of neutral sulfur in some of our experiments. The decrease in the total sulfur excretion shows that the neutral sulfur, which disappears from the urine, is not eliminated in any other form through this channel. Apparently, some sulfur-containing substance, which is ordinarily excreted in the neutral sulfur fraction of the urine, is involved in the catabolism of the substances which we have investigated.

SUMMARY

1. Isobarbituric acid, isodialuric acid, and formyloxaluric acid have been fed to dogs. Evidence was obtained that these compounds, when fed in small amounts, are to a great extent metabolized to yield urea.

2. The rise in the urinary ethereal sulfate output after feeding isobarbituric acid seems to indicate that this substance is partly excreted in conjugation with sulfuric acid. This view is supported by the preliminary studies made on a compound isolated from the urine of dogs after the feeding of isobarbituric acid.

3. The three above compounds have a distinct effect on the sulfur metabolism of the dog. A drop in the neutral sulfur fraction of the urine was observed in every case after feeding these substances. In some of our experiments there was no detectable amount of neutral sulfur excreted.

4. The assumption is made that in the catabolism of isobarbituric acid, isodialuric acid, and formyloxaluric acid in the organism of the dog a sulfur-containing substance is involved which is ordinarily excreted in the neutral sulfur fraction of the urine.

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STUDIES ON THE PHYSIOLOGY OF PYRIMIDINES

VI. THE FATE OF PARABANIC ACID, ALLOXAN, AND ALLOXANTIN IN THE ORGANISM OF THE DOG*

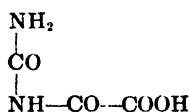
By LEOPOLD R. CERECEDO

WITH THE TECHNICAL ASSISTANCE OF ETHEL F. CURRY, JAKOB A. STEKOL,
AND HENRY ELBAUM

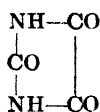
(From the Division of Biochemistry, University of California Medical School,
Berkeley)

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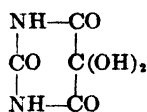
In a recent study (1) experiments were reported showing that oxaluric acid (I) might be conceived of as one of the intermediary metabolites of uracil. It was also pointed out that this substance might possibly be a link connecting the breakdown of purines and pyrimidines in the animal body. Inasmuch as parabanic acid (II) and alloxan (III) are obtained in the oxidation of uric acid by various oxidizing agents, it seemed of interest to reinvestigate the fate of these substances in the dog.



I



II



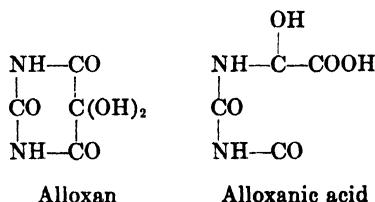
III

In a publication dealing with the metabolism of parabanic acid, Lewis (2) has summarized the literature on the subject. From the results of his own experiments this author concluded that no significant amounts of parabanic acid were broken down to urea in the dog or in the rabbit. He assumed that this compound was almost completely excreted unchanged, since he obtained, after the administration of the substance, a rise in the urinary total nitrogen output which was not accounted for by an increase in the urea plus ammonia nitrogen.

* This investigation has been aided by a grant from the National Research Council.

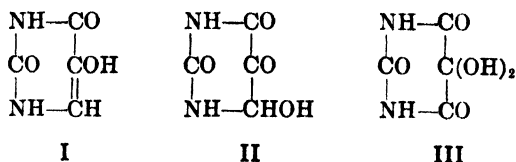
The physiological behavior of alloxan was studied by Koehne (3) who fed this substance in 8 gm. doses to dogs. He observed that it was to a great extent destroyed in the body. Lusini (4), on the basis of his observations on the fate of alloxan in metabolism, concluded that this compound was to a large extent completely oxidized, and that only a small fraction escaped destruction, being excreted in the urine in the form of alloxantin and parabanic acid.

The investigation of the metabolism of alloxan promised interesting results for the following reasons. In contact with alkali, alloxan is converted into alloxanic acid. This reaction may be represented as follows:



Felix (5) assumes, on the basis of his observations concerning the action of liver extracts on uric acid, that alloxanic acid is one of the intermediary products in the biological degradation of uric acid. He also considers the possibility that alloxanic acid may be further broken down in the body to urea.

Our attention was directed towards alloxan from still another angle. In a recent investigation (6) it was shown that isobarbituric acid and isodialuric acid have a distinct effect on the sulfur metabolism of dogs. Of particular interest in this connection was the observation that after feeding these compounds there was no detectable amount of neutral sulfur present in the urine. An examination of the formulæ represented below shows the structural relationships between isobarbituric acid (I), isodialuric acid (II), and alloxan (III).



The similarity in the constitution of these three compounds suggested that alloxan might influence the sulfur metabolism of the dog in the same manner as isobarbituric acid and isodialuric acid.

The third line of thought was as follows: The observations of Strecker (7), Wieland (8), and others have shown that alloxan may function as a hydrogen acceptor. This fact brought to mind the idea that in the oxidation of the above substances in the animal body we might be dealing with an oxidation-reduction process in which a sulfur-containing compound is involved. This conception gains some support from the observations of Dobrowolska (9) on the oxidation of uric acid in the presence of thioglycollic acid as a hydrogen acceptor. Still more significant in this connection are the recently recorded observations of Labes and Freisburger (10) concerning the action of cysteine on alloxan. These workers report that in this reaction alloxan is reduced to alloxantin, whereby cysteine is dehydrogenated.

The object of the experiments to be reported below was to study the fate of parabanic acid and alloxan in dogs.

EXPERIMENTAL

The parabanic acid and the alloxan used were commercial preparations. The purity of the compounds was checked by Kjeldahl nitrogen determinations. The experimental technique has been described in a previous communication (11). Oxalic acid was precipitated from the urine as calcium oxalate and this determined volumetrically.

Experiments with Parabanic Acid

In Table I are recorded the results obtained after feeding parabanic acid. An examination of the data reveals that this compound is not broken down to urea. We find in every case an increase in the total nitrogen and ammonia output on the day after feeding this substance. The difference between the rise in total nitrogen and the increase in ammonia nitrogen accounts only for about one-half of the amount of parabanic acid fed. Under Dogs 5 and 4 we have included the data for oxalic acid. Although we are conscious of the fact that the method used is inaccurate, we believe that the figures recorded have a relative significance. The pronounced rise in the amount of oxalic acid excreted seems to

indicate that parabanic acid is partly broken down in the organism of the dog. The increased output of oxalic acid accounts for the rise in the ammonia excretion.

TABLE I
Experiments with Parabanic Acid

	Day	Urine volume	Total N	Urea N	Ammonia N	Inorganic S	
		cc	gm.	gm	gm.	gm.	
Dog 3; weight 10.8 kilos	1	103	4 86	4 17	0 23	0.203	
	2	99	4 96	4 30	0.22	0.201	
	3	97	4 90	4 26	0 20	0.192*	
	4	115	5 42	4 24	0.36	0.198	
	5	115	5 12	4 34	0 27	0.206	
	6	110	5 07	4 31	0 24	0 205	
	7	111	5 21	4.40	0.21	0.209	
Dog 5; weight 13.0 kilos	1	135	5.24	4.50	0 23	0.230	Oxalic acid
	2	132	5.16	4 40	0.20	0 249	mg.
	3	160	5.24	4 55	0 22	0.240	4 7
	4	160	5 73	4.40	0 33	0.259	7.7*
	5	150	5.30	4.50	0 29	0 259	70 0
	6	160	5.38	4 64	0 27	0.261	12.0
	7	140	5.36	4 66	0.27	0 254	9 5
Dog 4; weight 11.4 kilos	1	150	5.34	4.41	0.45	0.188	7 2
	2	157	5.26	4 27	0.49	0.181	9.7
	3	149	5.29	4.40	0 40	0.185	38.3†
	4	169	5.66	4.43	0 53	0 184	17 5
	5	157	5.34	4.48	0.41	0.166	13.1
	6	147	5.21	4.32	0.43	0 178	
	7	155	5.28	4.35	0.46	0.179	

* 3.0 gm. of parabanic acid fed with food; N = 0.74 gm.

† 2.5 gm. of parabanic acid fed with food; N = 0.61 gm.

Isolation of Parabanic Acid from Urine

In view of the above results, it was deemed of interest to attempt to isolate the parabanic acid from the urine after feeding this compound. Fosse (12) has shown that parabanic acid con-

denses with xanthidrol to form dixanthidrylparabanic acid. Dixanthidrylparabanic acid melts at 222–223°.

Before attempting to isolate parabanic acid in our feeding experiments, we tested the method on urine to which this substance had been added. The procedure was as follows:

0.25 gm. of parabanic acid was dissolved in 50 cc. of dog urine (filtered). Basic lead acetate was added until no more precipitate formed. After standing for several hours, the lead precipitate was filtered and washed with water. The residue remaining on the filter was suspended in 50 cc. of water and treated with hydrogen sulfide. The lead sulfide was filtered off and washed thoroughly. The filtrate was concentrated *in vacuo* at 30–40° to a volume of 12 cc. To the residue 8 cc. of glacial acetic acid were added, and this mixture was treated with a solution of 0.5 gm. of xanthidrol in 24 cc. of glacial acetic acid. A white precipitate formed immediately. After standing for 2 hours, the substance was filtered. An analysis of the dried compound yielded the following results.

$C_{29}H_{18}O_8N_2$. Calculated, N 5.90; found, N 5.44

These findings justified the attempt to isolate the parabanic acid from the urine of a dog which had ingested 2.5 gm. of parabanic acid. The 24 hour sample of urine voided by the animal after the feeding of this compound was concentrated to a volume of 100 cc. Using the procedure described above, we were able to isolate from the urine a small amount of parabanic acid in the form of its dixanthidryl derivative, which melted at 216°. On analysis the compound yielded the following results:

$C_{29}H_{18}O_8N_2$. Calculated, N 5.90; found, N 6.47, 6.39

These findings show that parabanic acid was partly excreted unchanged in the urine.

Experiments with Alloxan

In Tables II and III we present the data obtained after feeding alloxan monohydrate. In all the experiments but one we observed a slight increase in the total nitrogen output. The urea figures show that alloxan is not broken down to urea in the organism of the dog. In every case we find a drop in the inorganic sulfur output on the day after the administration of this substance.

Corresponding to this decrease, we find less total sulfur eliminated (Table III). Contrary to our expectations, the neutral sulfur excretion does not undergo any change after the ingestion of alloxan. The ethereal sulfate fraction remains also unaffected. We believe that these findings justify the assumption that some of the alloxan is combined in the body with sulfuric acid and excreted through channels other than the kidney, possibly in the bile. In every case we observed that the dog voided a red-colored

TABLE II
Experiments with Alloxan

Day	Dog 7; weight 9.0 kilos					Dog 3; weight 12.2 kilos				
	Urine volume	Total N	Urea N	Ammonia N	Inorganic S	Urine volume	Total N	Urea N	Ammonia N	Inorganic S
	cc	gm	gm.	gm	gm	cc	gm.	gm	gm	gm
1	125	5.14	4.33	0.32	0.319	115	5.13	4.31	0.35	0.222
2	101	5.07	4.33	0.32	0.328	130	5.04	4.19	0.34	0.222
3	115	4.92	4.18	0.37	0.323	119	5.02	4.24	0.29	0.236
4	85	5.06	4.24	0.39	0.319	119	5.08	4.23	0.29	0.239†
5	165	5.00	4.10	0.39	0.328*	196	4.99	4.05	0.25	0.194‡
6	175	5.15	4.15	0.42	0.290	185	5.23	4.30	0.34	0.259
7	145	5.17	4.29	0.42	0.358	150	5.15	4.31	0.37	0.251†
8	135	5.18	4.33	0.44	0.335	170	5.21	4.10	0.33	0.207‡
9						173	5.23	4.34	0.35	0.262
10						155	5.22	4.33	0.33	0.252

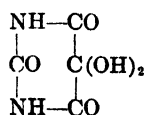
* 2.5 gm. of alloxan monohydrate fed with food; N = 0.44 gm.

† 3.0 gm. of alloxan monohydrate fed with food; N = 0.52 gm.

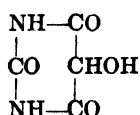
‡ Urine red.

urine after the ingestion of alloxan. Our interpretation of this fact is as follows:

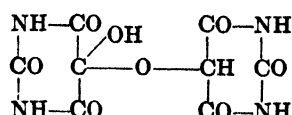
By an oxidation-reduction process part of the alloxan (I) is converted into dialuric acid (II). These two substances condense to form alloxantin (III). On combination with ammonia, alloxantin yields murexide, which causes the purple-red color of the urine.



I



II



III

We carried out qualitative tests for alloxantin on the urine and found them to be positive.

A similar observation has been recorded by Labes and Freisburger (10). These authors observed that mice, which had been poisoned with alloxan, also voided a red-colored urine.

TABLE III
Experiments with Alloxan

	Day	Urine volume	Total N	Urea N	In- organic S	Total S	Total sulfate S	Ethe- real S	Neutral S
		cc.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Dog 10; weight 11.6 kilos	1	220	4.10	3.25	0.299	0.330	0.317	0.018	0.013
	2	165	4.24	3.41	0.304	0.346	0.320	0.016	0.026
	3	125	4.39	3.45	0.303	0.340	0.320	0.017	0.020*
	4	125	4.64	3.41	0.273	0.314	0.288	0.015	0.026†
	5	125	4.60	3.63	0.320	0.365	0.337	0.017	0.028
	6	120	4.51	3.57	0.303	0.348	0.321	0.018	0.027
	7	150	4.37	3.39	0.305	0.349	0.319	0.014	0.030
	8	120	4.20	3.29	0.306	0.353	0.330	0.024	0.023

	Day	Urine vol- ume	Total N	Urea N	Am- monia N	In- organic S	Total S	Total sulfate S	Ethe- real S	Neutral S
		cc.	gm	gm	gm.	gm.	gm.	gm.	gm	gm.
Dog 7; weight 9.1 kilos	1	118	5.05	4.21	0.43	0.323	0.365	0.343	0.020	0.022
	2	139	5.17	4.31	0.44	0.322	0.384	0.347	0.025	0.037
	3	125	5.10	4.26	0.44	0.320	0.385	0.348	0.028	0.037*
	4	139	5.29	4.12	0.44	0.298	0.364	0.317	0.019	0.047†
	5	120	5.26	4.19	0.42	0.318	0.369	0.339	0.021	0.030
	6	137	5.23	4.32	0.45	0.336	0.400	0.356	0.020	0.044

* 2.5 gm. of alloxan monohydrate fed with food; N = 0.44 gm.

† Urine red.

Experiments with Alloxantin

The above observations suggested the idea of investigating the behavior of alloxantin in the dog. The fate of this compound in metabolism was studied by Wöhler and Frerichs (13). They fed 5 to 6 gm. doses of alloxantin to men and found that it was broken down in the body to urea. Koehne (3) administered the same substance to dogs and observed that it was completely oxidized. Lusini (4) inferred from his observations that alloxantin was

partly destroyed in the body and partly excreted in the form of murexide.

Alloxantin was prepared according to the directions given by Fischer (14).

The results obtained after feeding alloxantin are recorded in Table IV. The data show also in this case a distinct drop in the inorganic sulfur output on the day following the administration of the compound. The figures also demonstrate a decrease in the urea and total nitrogen excretion. This may have been due to a slightly toxic effect of the substance on the kidney. The dog voided a red-colored urine after the ingestion of the compound. An examination of the urine for alloxantin showed this substance to be present.

TABLE IV
Experiments with Alloxantin

	Day	Volume	Total N	Urea N	Ammonia N	Inorganic S
		cc.	gm.	gm	gm	gm
Dog 7; weight 9.0 kilos	1	135	5 10	4 33	0 38	0 340
	2	123	5.15	4.30	0 42	0.340
	3	139	5.22	4 40	0.41	0.342*
	4	125	4.79	4.22	0 38	0.241†
	5	135	5 22	4.21	0 52	0.385
	6	129	4.99	4 20	0.36	0 323
	7	125	5.00	4 15	0 39	0.326

* 2.5 gm. of alloxantin fed with food; N = 0.49 gm.

† Urine red.

DISCUSSION

The findings reported above indicate that parabanic acid, when fed to dogs, is to a great extent excreted unchanged. A small fraction of this substance is broken down in metabolism to yield oxalic acid.

As regards alloxan, our findings do not support the idea expressed in the introduction concerning this compound. The similarity in structure of alloxan, isobarbituric acid, and isodialuric acid had led us to assume that alloxan might have an effect on the neutral sulfur fraction of the urine, similar to that shown by isobarbituric acid and isodialuric acid. We found only a drop in the inorganic sulfur fraction, which suggests that alloxan is to a great extent excreted in the bile as an ethereal sulfate. The ob-

servation that alloxan is partly converted into alloxantin is in agreement with the findings recorded by Lusini (4).

We believe that, in connection with our previously (1) expressed idea concerning the intermediary metabolism of uric acid, the most important conclusion to be drawn from the observations recorded in the present investigation is the fact that alloxan and parabanic acid are probably not physiological breakdown products of uric acid.

SUMMARY

1. Parabanic acid, alloxan, and alloxantin have been fed to dogs.
2. Evidence was obtained that parabanic acid when administered to dogs is to a great extent excreted unchanged. The parabanic acid was isolated from the urine in the form of its dioxanthryl derivative. A small fraction of the compound is apparently converted into oxalic acid.
3. Following the feeding of alloxan a distinct drop in the output of inorganic sulfates was noted. Inasmuch as this decrease is not compensated by the rise of any other sulfur fraction of the urine, the assumption is made that alloxan is possibly partly excreted in the bile, perhaps as an ethereal sulfate. Evidence was obtained that a fraction of the alloxan is converted into alloxantin and excreted in the urine as murexide.
4. The experiments with alloxantin suggest that this substance is also possibly partly excreted in the bile and partly eliminated in the urine in the form of murexide.

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STUDIES ON PURINE METABOLISM

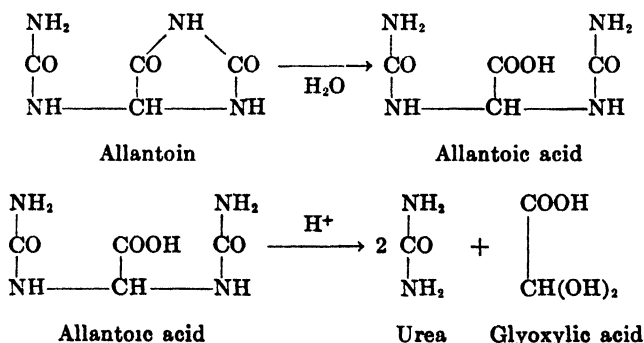
I. A NEW METHOD FOR THE DETERMINATION OF ALLANTOIN IN DOG URINE

BY FRANK WORTHINGTON ALLEN AND LEOPOLD R. CERECEDO

(From the Division of Biochemistry, University of California Medical
School, Berkeley)

(Received for publication, June 29, 1931)

Fosse, Brunel, and de Graeve (1) have recently described a new method for the determination of allantoin in urine. In their procedure the urine is treated first with a soy bean meal preparation which destroys the urea present and at the same time converts the allantoin into allantoic acid. In a second step the allantoic acid is broken down by acid hydrolysis to urea and glyoxylic acid. The reactions involved are as follows:



Urea is precipitated as dioxanthidryl urea, which is estimated gravimetrically. From the amount of urea formed, Fosse, Brunel, and de Graeve calculate the quantity of allantoin originally present in the sample analyzed. Their technique in our hands did not yield satisfactory results.

It is a well known fact that hydrolysis of allantoin with dilute alkali yields allantoic acid (2). We have used this reaction for

converting the allantoin into allantoic acid. The hydrolysis of allantoic acid to urea and glyoxylic acid was effected by means of dilute hydrochloric acid.

EXPERIMENTAL

After considerable experimentation the following method has been developed. The 24 hour sample of dog urine is diluted to 500 cc. To a 10 cc. sample of the diluted urine 5 cc. of 10 per cent urease (Squibb) are added. This mixture is incubated at 38° for 1½ hours. The protein of the urease is precipitated by

TABLE I
Recovery of Allantoin Added to Urine

	Sample No.	Allantoin added	Allantoin found	Allantoin calculated	Difference	Allantoin recovered
		mg.	mg.	mg	mg.	per cent
Experiment 1	1	0	10.367			
	2	1.0	11.370	11.367	+0.003	100.3
	3	2.0	12.490	12.367	+0.123	106.1
	4	3.0	13.532	13.367	+0.165	105.5
Experiment 2	1	0	9.998			
	2	1.0	11.013	10.998	+0.015	101.5
	3	2.0	12.003	11.998	+0.005	100.2
	4	3.0	13.150	12.998	+0.152	105.0
Experiment 3	1	0	8.265			
	2	1.0	9.267	9.265	+0.002	100.2
	3	2.0	10.293	10.265	+0.028	101.4
	4	3.0	11.259	11.265	-0.006	99.8

means of 2 cc. of Tanret's reagent and filtered off. 10 cc. of the filtrate are transferred to a 50 cc. volumetric flask. The solution is neutralized with N KOH with phenolphthalein as indicator. An excess of alkali is added until the concentration of base is 0.2 N. The mixture is kept for 2 hours on the water bath at 70°. Thus, the allantoin is hydrolyzed to allantoic acid. Normal HCl is then added until the acidity of the solution is 0.1 N. Warming the mixture at 70° for 30 minutes hydrolyzes the allantoic acid present to urea. After cooling, the solution is made up to volume. In 5 cc. samples the urea is determined according to the method described by Allen and Luck (3).

The above equations show that 158.09 gm. of allantoin yield on hydrolysis 120 gm. of urea. Hence the equivalents for the calculation are: 1 cc. of 0.1 N potassium dichromate is equivalent to 0.0518 mg. of urea, and 1 cc. of 0.1 N dichromate is equivalent to 0.0682 mg. of allantoin.

We have tested the method upon dog urine to which allantoin had been added with results which are shown in Table I. In all

TABLE II

Allantoin Output of Dog Maintained on Cowgill's Diet

Dog 7; weight 9.0 kilos.

Day	Volume	Total N	Urea N	Ammonia N	Allantoin N	Total allantoin	Inorganic S	$\frac{\text{Allantoin N}}{\text{Total N}} \times 100$
	cc	gm	gm.	gm.	gm.	gm.	gm	
1	85	4.84	3.97	0.37	0.115	0.325	0.366	2.4
2	90	4.80	4.03	0.37	0.119	0.336	0.360	2.5
3	85	4.64	4.00	0.37	0.107	0.303	0.352	2.3
4	90	4.64	3.92	0.37	0.120	0.337	0.352	2.6
5	90	4.66	4.00	0.33	0.109	0.306	0.349	2.3

TABLE III

*Allantoin Excretion on a Protein-Free Diet**

Dog 5; weight 12.5 kilos.

Day	Total N	Allantoin N	Total allantoin	$\frac{\text{Allantoin N}}{\text{Total N}} \times 100$
	gm.	gm.	gm.	
1	1.05	0.099	0.280	9.5
2	0.97	0.093	0.261	9.5
3	0.82	0.078	0.219	9.5

* 90 gm. of cane sugar and 45 cc. of Cowgill's fat mixture (4) daily.

cases the 24 hour sample of urine was made up to 500 cc., and 10 cc. samples of the diluted urine, as indicated in Table I, were analyzed.

For comparison we show in Table II the data obtained from the urine of Dog 7, which was kept on the synthetic diet recommended by Cowgill (4). The urine was collected by catheterization every 24 hours.

In Table III the data are recorded for the daily output of total nitrogen and allantoin of a dog kept on a protein-free diet. The analyses were carried out after the dog had been on a diet consisting of carbohydrate and fat for 2 weeks.

DISCUSSION

According to Fosse and his coworkers (5) *Soja hispida* contains, in addition to urease, allantoinase and uricase.

The allantoinase is supposed to transform allantoin into allantoic acid. The presence of uricase would give rise to allantoin if uric acid were present in the urine. We have analyzed human urine under the conditions specified above. We were unable to detect the presence of urea after completion of the analytical procedure.

We believe that we are justified in assuming that in the dilutions used in this method, the small amount of uric acid present in dog urine would not seriously interfere with the procedure.

In Table II we have included the value for the allantoin nitrogen to total nitrogen percentage ratio. An examination of the data reveals the constancy of this quotient under the conditions used in our experiments. For this dog the average $\frac{\text{allantoin N}}{\text{total N}}$ per cent ratio was 2.5. For Dog 5, which was kept on a protein-free diet, the ratio was found to be 9.5 (Table III).

SUMMARY

A method is described for the determination of allantoin in dog urine. The method is based on the fact that allantoin may be hydrolyzed by alkali to allantoic acid. Allantoic acid on acid hydrolysis yields 2 molecules of urea. Urea is precipitated as dioxanthidryl urea, which is estimated by oxidation with potassium dichromate and sulfuric acid.

The average allantoin nitrogen to total nitrogen percentage ratio in the urine of full grown dogs under the experimental conditions observed in the present investigation was found to be 2.5. For a dog kept on a protein-free diet the ratio was 9.5.

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TYRAMINE OXIDASE

II. THE COURSE OF THE OXIDATION

By MARY L. C. BERNHEIM*

(From the Department of Biochemistry, Duke University School of Medicine, Durham)

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A few years ago I was able to show (1) that the livers of most animals are able to effect the oxidative deamination of tyramine. Under the conditions which then obtained tyramine was found to take up 1 atom of oxygen per molecule and to liberate as ammonia 50 per cent of its nitrogen content. This reaction has been further studied and it has been found that the course of the oxidation varies greatly under different conditions.

Ewins and Laidlaw (2) isolated the corresponding acid (*p*-hydroxyphenylacetic acid) from a solution of tyramine which had been perfused through the surviving liver. This seemed therefore the most probable end-product of the reaction. But the oxygen necessary for the production of the acid represents an uptake of 2 atoms per molecule of tyramine and the complete liberation of the nitrogen. It has now been found that, given suitable conditions, an enzyme in liver can oxidize tyramine to the acid, and in certain cases the oxidation can be carried even further. It seems likely that at least three distinct processes are involved in the oxidation of tyramine.

EXPERIMENTAL

The technique used for the estimation of oxygen absorption is that described by Hare except that the constant volume Barcroft-Warburg manometer replaced the differential type of manometer previously used. Guinea pig liver was chopped and ground with sand and an equal volume of buffer and then squeezed through

* Research Fellow of Newnham College, Cambridge, England.

muslin. A quantity of the preparation was placed in one of the vessels. A solution of tyramine hydrochloride in buffer was placed in the side arm of the vessel, and when temperature equilibrium was reached the vessel was closed and the two substances were mixed. A similar vessel contained an equal amount of liver preparation, and buffer or water instead of the tyramine. Buffer was added to the vessels to make the total volume of the liquid 2 cc. Each vessel contained a small vessel inside it with 20 per cent sodium hydroxide to absorb the carbon dioxide, and the uptake of oxygen due to the tyramine was calculated by subtracting the uptake of the liver alone from that of the liver and tyramine together. Temperature and pressure corrections were made in the usual way. The experiments were performed at a temperature of 30°.

Effect of pH

It has been previously shown that the oxidation proceeds most rapidly in alkaline solution and that the optimum pH is about 9.9. This was determined by measuring the oxygen uptake during the first half hour or hour of the experiment. If, however, the reaction is allowed to continue to completion, it is found that the amount of oxygen taken up is dependent on the pH. In neutral and alkaline solutions the reaction stops when the equivalent of 1 atom of oxygen has been taken up, but in acid solutions it proceeds until the equivalent of 4 atoms of oxygen has been absorbed, although the oxidation in the acid medium is comparatively slow.

Experiment 1—A guinea pig was killed and the liver was taken immediately and finely chopped and ground with sand. It was then divided into two equal parts. One part was diluted with an equal volume of acetate buffer at pH 5.2 and the other part was diluted with phosphate buffer at pH 8.0. The buffers were made up in a concentration of 0.5 M. The pH of the reaction mixtures was tested colorimetrically at the beginning and end of the experiment and was found to remain constant. Vessel 1 contained 1 cc. of liver preparation pH 5.2, 0.5 cc. (1 mg.) of tyramine hydrochloride, and 0.5 cc. of buffer pH 5.2. Vessel 2 contained the same except that 0.5 cc. of water was used instead of tyramine. Vessels 3 and 4 were identical with Vessels 1 and

2 respectively, except that the pH was 8.0. Curves A and B, shown in Fig. 1, were obtained by subtracting the uptake shown by Vessel 2 from that of Vessel 1 and similarly by subtracting the uptake of Vessel 4 from Vessel 3.

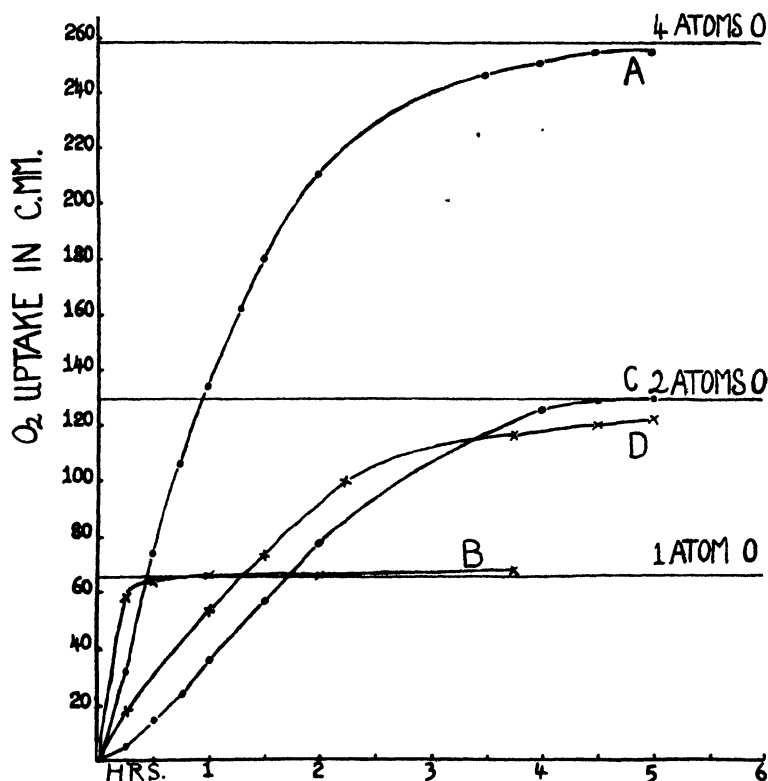


FIG. 1. Oxidation of 1 mg. of tyramine hydrochloride. The horizontal lines represent the theoretical uptake for the oxidation of 1 mg. of tyramine hydrochloride by 1, 2, and 4 atoms of oxygen. Curve A = 1 cc. of liver preparation, pH 5.2; Curve B = same at pH 8.0; Curve C = 0.25 cc. of liver preparation, pH 5.2; Curve D = same at pH 8.0.

Effect of Concentration

In alkaline solutions, concentrated liver preparations oxidize tyramine to the equivalent of 1 atom of oxygen and then stop. But with diluted liver preparations the oxidation continues, some-

times as far as 2 atoms of oxygen but never further. This would suggest the presence of a system responsible for the cessation of the oxidation at 1 atom of oxygen. If insufficient liver is present, the concentration of this system is too low to enable it to be effective, and the oxidation continues and tends toward the absorption of 2 atoms of oxygen. In acid solution, as shown in Experiment 1, the concentrated liver preparation oxidizes tyramine with an uptake of 4 atoms of oxygen. Dilution of the liver preparation results in a slowing of the reaction and a decrease in the amount of oxygen absorbed. The system reaches equilibrium at a point between that corresponding to an absorption of 4 atoms and that corresponding to 2 atoms. Very dilute liver preparations at pH 5.2 can absorb the equivalent of 2 atoms of oxygen but no more. By the same reasoning as that given above, this indicates that the liver is still able to bring about the oxidation of tyramine, but that the further oxidation (to 4 atoms) is dependent on a separate system, which is less stable, or present in smaller quantities, than the other. It is thus possible, by means of suitable dilutions, to obtain sufficient enzyme for the first process, whereas the other catalyst is present in such small amounts as to be ineffective. Curves C and D, Fig. 1, show a typical experiment.

Effect of Age of Liver Preparation

It seemed likely, from the data given above, that the oxidation of tyramine by 2 atoms of oxygen, corresponding to the production of the acid, *p*-hydroxyphenylacetic, is the most stable of the systems involved. If this is so the other two systems, which cause the oxygen uptake to reach 4 atoms or to stop at 1 atom respectively, should tend to be destroyed first when the liver preparation is allowed to stand. This was actually found to be the case. When the liver preparation remains under aseptic conditions at 0° for 24 to 48 hours it is found that the extra oxygen uptake in acid disappears completely and the system reaches equilibrium when exactly 2 atoms of oxygen have been taken up. Similarly on the alkaline side, the oxidation no longer stops when 1 atom of oxygen has been taken up, but continues and stops at the equivalent of 2 atoms of oxygen per molecule of tyramine. Fig. 2 shows one of many experiments. In all cases the liver preparation was made up in buffer at the required pH and allowed to stand at that pH.

Effect of Potassium Cyanide and Sodium Pyrophosphate

It has been shown by Hare that cyanide present in a concentration which is sufficient to inhibit most oxidative enzyme sys-

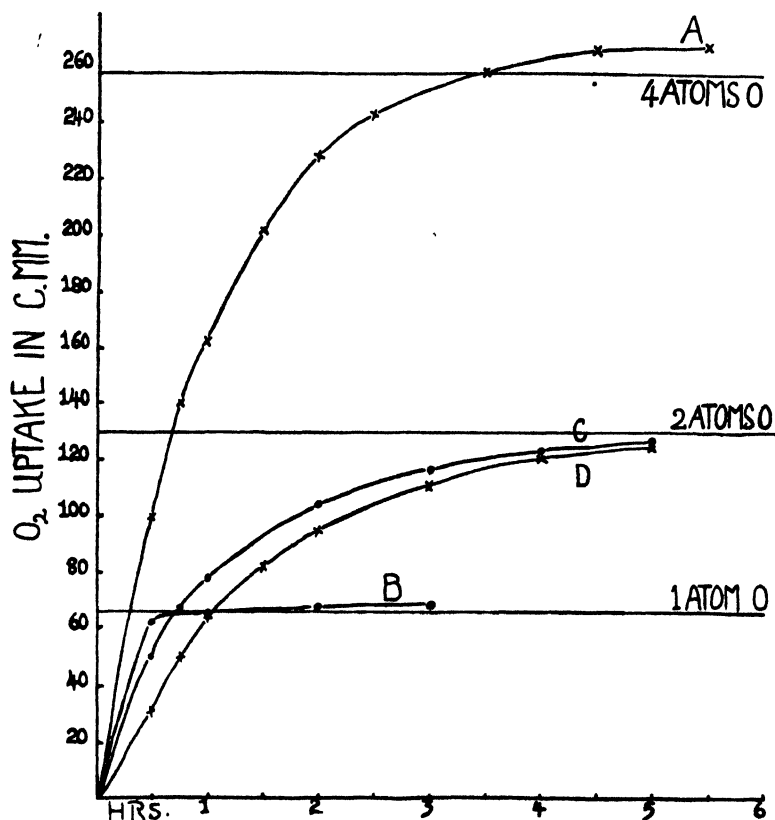


FIG. 2. Effect of the age of the liver preparation. The horizontal lines represent the theoretical uptake for the oxidation of 1 mg. of tyramine hydrochloride by 1, 2, and 4 atoms of oxygen. Curve A = fresh liver preparation, pH 5.2; Curve B = same at pH 8.0; Curve C = liver preparation 36 hours old, pH 8.0; Curve D = same at pH 5.2.

tems has no effect upon the tyramine oxidase. The alkaline oxidation to 1 atom of oxygen continues unchanged in the presence of 0.005 M KCN. It was found, however, that with older liver preparations cyanide inhibits the oxidation to 2 atoms of oxygen

which normally takes place, and therefore alkaline preparations 24 to 48 hours old behave in the presence of cyanide as if they were fresh. They take up the equivalent of 1 atom of oxygen per molecule of tyramine. The effect of sodium pyrophosphate

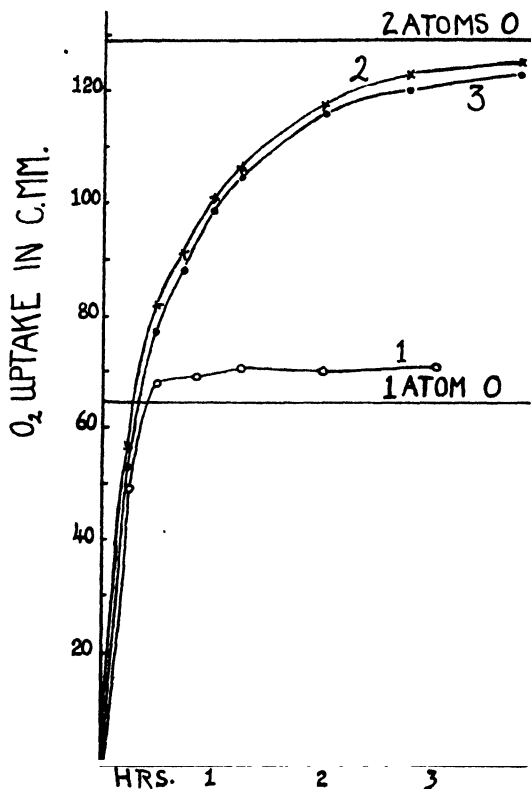


FIG. 3. Effect of KCN and sodium pyrophosphate on the oxidation of 1 mg. of tyramine hydrochloride by 36 hour old liver. The horizontal lines represent the theoretical uptake for the oxidation of 1 mg. of tyramine hydrochloride by 1 and 2 atoms of oxygen. Curve 1 = plus KCN 0.005 M; Curve 2 = plus sodium pyrophosphate 0.05 M; Curve 3 = control.

was then tried, for it should show an inhibition similar to that of KCN if the latter effect is due to the removal of traces of iron. The presence of 0.05 M pyrophosphate, however, did not influence any of the reactions. The inhibition by cyanide is therefore thought

to be due to a specific effect on one of the processes of the tyramine oxidation and is not due to the removal of traces of iron.

Experiment 2—Fresh guinea pig liver was ground with sand and phosphate buffer, pH 8.0, and allowed to stand at 0° for 36 hours. Six Warburg vessels were taken and in each was placed 0.5 cc. of the liver preparation. To Vessel 1 were added 0.25 cc. (1 mg.) of tyramine hydrochloride (in the side arm) and 0.5 cc. of 0.2 M sodium pyrophosphate, pH 8.0. To Vessel 2 was added 0.5 cc. of pyrophosphate but no tyramine. Vessel 3 received 0.25 cc. of tyramine hydrochloride, and Vessel 4 liver and buffer only. Vessels 5 and 6 received 0.2 cc. of 0.05 M KCN and Vessel 5, 0.25 cc. of tyramine hydrochloride. To all the vessels phosphate buffer was added to bring the total volume up to 2 cc. The results are shown in Fig. 3. Further, it can be shown that with very dilute fresh alkaline liver preparations, such as show an oxygen uptake of more than 1 atom per molecule of tyramine, the presence of KCN inhibits the extra oxygen uptake which is obtained by dilution and the uptake stops at 1 atom.

When the oxidation in acid solution in the presence of KCN was investigated it was found that 0.005 M KCN shows a definite inhibition of the reaction, which increases as the reaction proceeds; the final uptake may be anywhere between 2 and 4 atoms of oxygen per molecule of tyramine, and the curves which are obtained suggest a gradual destruction of the enzyme. Sodium pyrophosphate has no effect on the reaction. Acid preparations 24 hours old show about the same percentage inhibition with KCN as the fresh liver, and no inhibition with pyrophosphate.

Isolation of p-Hydroxyphenylacetic Acid

An attempt was now made to prove whether, when the equivalent of 2 atoms of oxygen has been taken up, *p*-hydroxyphenylacetic acid is formed from tyramine. Ewins and Laidlaw first showed that this change takes place almost quantitatively when tyramine is perfused through the surviving liver. However, they failed to find the acid after the incubation of minced liver and tyramine and were able to recover most of the tyramine unchanged. When a mixture of tyramine and liver is stirred or air is passed through it, if the conditions are suitable it can be shown that an oxygen uptake correspond-

ing to 2 atoms per molecule of tyramine takes place, and the mixture shows an increased ammonia content corresponding to the nitrogen in the tyramine. *p*-Hydroxyphenylacetic acid can be isolated from the mixture. But if tyramine and the same liver extract are allowed to stand at room temperature and not stirred, less than 10 per cent deamination occurs after several hours, and the tyramine can be recovered. It is suggested that the failure of Ewins and Laidlaw to obtain the acid from tyramine and liver was due to lack of suitable conditions, particularly of adequate aeration.

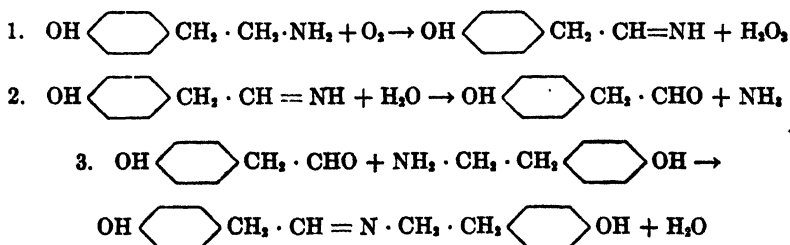
The process used for the isolation and identification of *p*-hydroxyphenylacetic acid was as follows: Pig liver was minced and mixed with an equal volume of 2 per cent sodium fluoride solution to hinder bacterial growth, squeezed through muslin, and allowed to stand at 0° for 24 hours. To 250 cc. of this extract was added an equal volume of phosphate buffer pH 7.4, in which 500 mg. of tyramine hydrochloride had been dissolved. The mixture was stirred at 30° with an electric stirrer and the amount of ammonia liberated from the tyramine was estimated at intervals by the method previously described by Hare. When complete deamination was obtained, the mixture was slightly acidified with acetic acid and boiled for 5 minutes. This destroys the enzyme and removes most of the liver protein, which can be filtered off. The precipitate was washed several times until the washings gave only a faint Millon reaction. The filtrates were combined and concentrated *in vacuo* until the volume was about 500 cc. This was placed in a thin walled collodion sac and dialyzed against three changes of water of 500 cc. each. Toluene was added every few hours to prevent bacterial decomposition, and after 36 hours the Millon reaction showed that the substance responsible for it had almost completely dialyzed. The dialysates were combined, evaporated to 200 cc., rendered slightly acid, and extracted in a continuous extractor with ether. The ether on evaporation left a brown crystalline mass. This was extracted many times with boiling benzene which yielded on cooling crystals of an acid giving a vivid Millon reaction. After recrystallization the melting point of this acid was 147°, which was not altered by mixing with a sample of *p*-hydroxyphenylacetic acid. This experiment was repeated twice and yields of 64 per cent and 72 per cent of the theoretical were obtained.

DISCUSSION

From what has been shown above it seems that the oxygen uptake obtained when tyramine and liver extract are mixed cannot be due to a single enzyme system. It is not possible to explain the several different oxidations which occur unless the existence of at least three systems is postulated.

Firstly, there is a system producing the oxidation of 1 molecule of tyramine by 4 atoms of oxygen. This occurs only in fresh liver and in an acid medium. The end-product of this system is at present unknown, but the possibility suggests itself that the oxidation follows a course comparable to that found by Raper (3) to occur in the case of the enzyme tyrosinase. *p*-Hydroxyphenylacetic acid, which is presumably first formed, must be further oxidized perhaps by the introduction of an oxygen atom into the ring in the ortho position with regard to the hydroxyl group already there. This compound would then form a quinone, as Raper has shown to occur in the production of melanin from tyrosine. This process would correspond to an uptake of 4 oxygen atoms per molecule of tyramine. It should be noted that the tyrosinase system is quite distinct from the tyramine oxidase, for tyrosinase does not occur in liver and the tyramine oxidase does not give rise to pigmented products.

Secondly, there is the system which involves the absorption of 1 atom of oxygen per molecule of tyramine. This is favored by an alkaline reaction. It is suggested as a possibility that a condensation takes place between 1 oxidized molecule of tyramine and 1 unoxidized molecule. It is probable that an aldehyde is first formed when deamination takes place. This aldehyde, instead of being further oxidized to the acid, might condense with the amino group of an available tyramine molecule to form a Schiff's base. The possible stages are shown below.



This theory is strengthened by the findings previously reported that during the reaction hydrogen peroxide is formed and that only 50 per cent of the nitrogen in the tyramine molecule can be found as ammonia.

The third process, represented by the absorption of 2 atoms of oxygen per molecule of tyramine, is the most stable of the three systems, and the one which survives when the others have been destroyed. Both dilution and ageing of the liver preparations, in either acid or alkaline solutions, eventually give this type of oxidation. It has been shown that the process involves the oxidation of the tyramine to *p*-hydroxyphenylacetic acid probably through the aldehyde. The first stages of the reaction are probably those represented in Equations 1 and 2, and the aldehyde is then oxidized directly to the acid.

The effect of the addition of potassium cyanide to the reaction mixture is interesting. The fact that sodium pyrophosphate has no effect on the reaction shows that the latter is not dependent upon the presence of traces of iron. In the alkaline reaction, KCN appears to prevent the oxidation of the aldehyde, or to encourage the condensation process. It is known, however, that KCN only very slightly inhibits the oxidation of aldehydes by the Schardinger enzyme which is present in liver (Dixon and Thurlow (4)), therefore the latter alternative seems more likely. Von Miller and Plöchl (5) have shown that KCN can combine with a Schiff's base to form the nitrile, but it is not possible to tell whether this reaction could take place under the present conditions. With regard to the reaction in an acid medium, the KCN effect is probably due to the slow destruction of an enzyme by the cyanide, but we have no means of deciding which system is attacked.

It was previously shown that under certain conditions only 50 per cent of the nitrogen was given off as ammonia. It has further been proved that, as would be expected, conditions involving the production of the corresponding acid involve at the same time the complete deamination of the tyramine. But the exact relationship of the liberation of ammonia to the oxidation processes has not yet been completely worked out, and it is hoped that it will form the subject of a later communication.

SUMMARY

1. It is shown that, under different conditions, different amounts of oxygen are taken up when tyramine is oxidized by liver; probably three systems are present.
2. The effect of pH, the age, and concentration of the liver preparation have been studied.
3. Under certain conditions, *p*-hydroxyphenylacetic acid is formed, and has been isolated.

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OXIDATIONS INDUCED BY SUGARS

I. THE FORMATION OF BARIUM PEROXIDE*

By PHILIP A. SHAFFER AND BEN K. HARNED

(From the Laboratory of Biological Chemistry, Washington University School of Medicine, St. Louis)

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It is well known that in the oxidation of many "auto oxidizable" substances by air or oxygen an activation of the oxygen occurs. This is evidenced by the fact that the oxidation of other substances may be thus induced, which are not themselves directly attacked by molecular oxygen. Many examples of induced oxidations have been studied and many views advanced as to their mechanism since the work of Schönbein (1840-1858) and Kessler (1863) who first noted such phenomena. The subject is well reviewed by Mellor (1909) who cites the chief contributions up to that time. More extended treatment is found in the monographs of Engler and Weissberg (1904) and Kastle (1909).

The logical explanation of this type of induced oxidations would appear to be that put forward by Bach, Engler, Manchot, Bodländer, and others, and established for the case of benzaldehyde by Baeyer and Villiger; namely, that the first product is a combination of the autoxidizable substance with a molecule of oxygen, the compound being a peroxide or "moloxide" (Traube) which may be a much more active oxidant toward many substances than is molecular oxygen. One evidence for this view is the well known fact that under certain conditions H_2O_2 is a chief and presumably the primary product of reaction between H_2 and O_2 .

* The data of this and later papers in this series are taken in part from a dissertation presented by Ben K. Harned to the Board of Graduate Studies in partial fulfilment of the requirements for the degree of Doctor of Philosophy, Washington University, June, 1929.

A preliminary report of this work was made before the Twenty-first annual meeting (1927) of the American Society of Biological Chemists.

With benzaldehyde on the other hand the primary product is very probably benzoyl hydrogen peroxide (perbenzoic acid) $C_6H_5CO \cdot O \cdot OH$ (Bodländer, Baeyer and Villiger). Both of these and other peroxides are much more active oxidants than molecular oxygen, and become very active indeed in the presence of catalysts like ferrous and certain other metal ions.

From the fact that molecular oxygen is the ultimate oxidant in virtually all biological oxidations, the possibility has long been appreciated that an induced activation of oxygen may occur in air-breathing cells. Indeed, so general is peroxidation when oxygen reacts with spontaneously oxidizable substances *in vitro*, that the same may reasonably be inferred to occur in respiring cells unless they possess some special device to avoid peroxide formation. In the latter case the nature of such a device would be of much interest.

According to the "dehydrogenation" hypothesis developed by Wieland and Thunberg, peroxidation is a factor in biological systems and (following the scheme of Traube) the peroxide formed is hydrogen peroxide, for which the enzyme catalase represents a protective device. From analogy with many organic substances in simple solution it seems probable that peroxides of the organic molecule may also be formed, and these are apparently not acted upon by catalase.

In the light of the facts above sketched it is not surprising to find that when glucose and other reducing sugars, dissolved in alkaline solution (in which they become oxidizable), are aerated or shaken with air or oxygen, peroxides are formed and the oxidation of various substances not oxidizable by molecular oxygen may be thus induced. Although this fact obviously does not justify the inference that oxidations are similarly induced during sugar oxidation in living cells, the possibility is indicated, and as above suggested may even be expected unless prevented by special inhibiting devices such as *intervening* reversible oxidation catalysts, the necessary behavior of which, in this respect, we hope to discuss in a later paper. In any case, oxygen activation by sugars has an intrinsic interest apart from possible biological applications.

Although the oxidation of sugars, induced by other oxidations has been observed, the only references we have found to oxygen activation as the *result* of sugar oxidation are the reports of Radziszewski (1876) and Bach (1897). Radziszewski observed that when aerated in alcoholic KOH, a large number of organic sub-

stances (aldehydes, fats, waxes, etc.) caused a phosphorescence. He stated, "Unter analogen Verhältnissen leuchtet auch Traubensucker, nur ist das Licht ziemlich schwach." Subsequently, he demonstrated that this phosphorescence is accompanied by an activation of oxygen as indicated by an induced oxidation of indigo which is only slowly affected by molecular oxygen.

Bach published a list of some forty substances, including glucose, which formed peroxides on slow oxidation. He gave practically no data, but suggested that similar peroxides may be responsible for many secondary oxidations in the animal body.

To these citations should perhaps be added the papers from this laboratory (Shaffer, 1921; Shaffer and Friedemann, 1924) on the oxidation of acetoacetate by H_2O_2 in the presence of glucose and other sugars,—the ketolytic action of sugars. Although the interpretation there given for this reaction was based on the idea of a condensation between the sugar and acetoacetate, it is not unlikely that activation of H_2O_2 through the intermediate formation of a sugar peroxide is also involved, and may even be the more important factor.

Many examples could be cited of the formation of organic peroxides or per-acids on treatment with H_2O_2 , the products having similar properties to the peroxides formed by the addition of molecular oxygen. The two phenomena—activation of O_2 and of H_2O_2 —are probably fundamentally similar. In both there is, in some cases, evidence also of a reaction between inductor (primary reductant) and acceptor (secondary or induced reductant), as well as between primary reductant and primary oxidant. Such evidence will be cited at the appropriate place, in later papers of this series.

In this paper we report experiments concerning the formation of peroxide, particularly crystalline barium peroxide, by the aeration of glucose and other sugars dissolved in alkaline solution. We shall later consider the oxidation of other substances, induced by sugars.

The formation of peroxide in aerated sugar solutions is very simply demonstrated as follows: A solution of about 0.5 per cent glucose in 0.5 to 1 *N* NaOH (or KOH) is vigorously aerated at room temperature. At intervals samples of the solution are removed and run into an excess of H_2SO_4 . On the addition of a few cc. of 5 per cent KI and soluble starch, the solution slowly becomes blue,

due to liberation of iodine. No blueing occurs before aeration, but becomes positive even after a few minutes of aeration, and increases with later samples during 24 hours or longer. The amount of peroxide is small and the color development slow, but becomes quite rapid (complete in about 5 minutes) on adding to the acidified sample a few drops of molybdic acid or ammonium molybdate or phosphomolybdic acid solution. Any of these so catalyze the reaction between KI and H_2O_2 (Brode, 1901) that exceedingly dilute solutions of hydrogen peroxide may be thus accurately determined by thiosulfate titration. (Without the catalyst very dilute solutions of H_2O_2 react very slowly with iodide.)

The amount of peroxide present in aerated sugar solutions may be illustrated by data from an experiment with 0.5 per cent glucose in 0.5 M NaOH at about 30° . After about an hour of aeration, the iodine liberated (by molybdic acid catalysis and titration with thiosulfate) corresponded to 0.0003 M peroxide, rising to about 0.002 M after about 24 hours. The latter quantity is roughly about 2 per cent of the total amount of oxygen absorbed for complete oxidation of the sugar under these conditions.

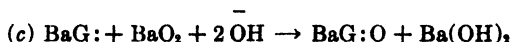
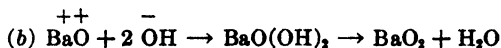
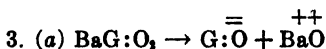
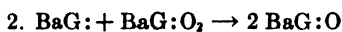
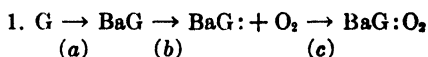
Another evidence of combined and evolvable oxygen in aerated sugar solutions is observed with glucose (and fructose) solutions in NaOH during oxidation by O_2 in the Barcroft-Warburg manometer vessels. If after an hour or so of oxygen absorption a suspension of MnO_2 is dumped from the side cup into the alkaline sugar solution, gas is quickly *evolved* for a short period, after which absorption is resumed (at a more rapid rate, due to Mn catalysis). The evolved gas is O_2 from H_2O_2 present in the sugar solution. (Experiments on these points, made for one of us by Miss Ellen Ehrenfest will be reported later.)

The most convincing evidence of peroxide formation is the precipitation of crystalline barium peroxide on shaking glucose (or fructose or lactose) dissolved in $\text{Ba}(\text{OH})_2$ solution with oxygen, a procedure suggested by the observation by Manchot and Herzog of BaO_2 formation on aeration of leucoindigo in $\text{Ba}(\text{OH})_2$. The precipitate has the crystal form of barium peroxide and on acidification with H_2SO_4 yields a solution giving all reactions of hydrogen peroxide, including the chromic and titanous acid tests.

Barium peroxide is formed with glucose only under somewhat

limited conditions. It is necessary that the ratio of dissolved O_2 to rate of sugar activation by alkali be high enough to avoid consumption of the peroxide faster than its formation. Some secondary consumption of peroxide by reaction with other active sugar molecules perhaps always occurs, since the yield of barium peroxide never reaches the maximum of one-half the total oxygen absorbed, observed with some other induced oxidations. This side reaction is minimum with low sugar concentration, high oxygen tension (rapid shaking), high alkalinity, and high Ba concentration.

We have not been able to demonstrate directly a peroxide other than H_2O_2 ; though from analogy with benzoyl hydrogen peroxide formed by oxygenation of benzaldehyde, and from the fact, to be reported in a later paper, that acceptors like indigo and acetoacetate are oxidized, when induced by sugar, at a rate greater than their oxidation by H_2O_2 , we are led to suppose that the hydrogen peroxide precipitated in the presence of Ba is a secondary product by reaction with water and alkali and that the primary product is a sugar peroxide. High alkalinity favors the conversion of organic peroxides into hydrogen peroxides, and acidity the reverse. The following scheme represents the reactions as we conceive them. G represents the sugar.



Stage (a) of Reaction 1 represents salt formation of the sugar (rapid), (b) a slow enolization of the sugar salt to an active form with exposed electron pairs, and (c) the oxygenation and peroxide formation. The rate of (c), oxygen being in excess, is limited by (b); i.e., by the concentration of active sugar $BaG:$. The hypothetical sugar peroxide $BaG:O_2$ is consumed by (Reaction 2) oxidation of sugar, and by (Reaction 3 (a)) formation of barium peroxide. The barium peroxide may be consumed by oxidation of active

sugar (Reaction 3 (c)); no peroxide will be evident if the rate of this reaction exceeds the combined rates of sugar peroxide formation (Reaction 1) and its reaction with sugar (Reaction 2). Although manifestly hypothetical as well as schematic, this outline appears to represent the facts so far as now known.

Several series of experiments were carried out to learn the conditions influencing the amount of barium peroxide formed in relation to the amount of sugar oxidized and to the total oxygen absorbed. Most of the experiments were performed with glucose. The chief factors besides rate of shaking (*i.e.*, rate of O₂ supply) appear to be alkalinity, sugar concentration, barium concentration, and temperature. The effects of the presence of methylene blue and of powdered glass were also studied.

Methods

Oxygen Absorption—Immediately after mixing the sugar and barium hydroxide solutions, 35 cc. of the mixture were measured into each of a series of calibrated 250 cc. bottles. These had been filled previously with oxygen from a cylinder. Each bottle was closed by a rubber stopper bearing a capillary tube drawn to a fine point. The tip of the capillary was sealed in a flame and the stopper covered with wax to prevent leakage.¹ The bottles were then placed in the carrier of a shaking device, wholly immersed in a large water bath, maintained at $37.5^{\circ} \pm 0.2^{\circ}$. By means of a motor-driven eccentric, revolving 80 times per minute, the carrier was moved back and forth through an arc of about 90° . At intervals the shaker was stopped for a moment and a bottle removed. The bottle was clamped in a 3 liter water bath, also at 37.5° , and immersed to the stopper. A gas burette (in a water jacket through which water circulated at 37.5°) was attached to the unbroken capillary tube of the reaction bottle by a short piece of small bore pressure tubing. After adjusting the gas burette and taking a zero reading, the end of the capillary was broken off within the rubber tubing thus connecting the gas inside the bottle with that in the burette. Equilibrium between the gas in the burette and bottle

¹ Some oxygen diffused from the bottle during the manipulations preceding sealing but introduced no error since our measurements involved a change in gas volume and not an analysis for oxygen. The carbon dioxide content of the oxygen or air was negligible in our experiments.

was established quickly, after which the final reading was recorded. The difference between the initial volume of the gas in the bottle and the volume after shaking, calculated as dry gas, standard conditions, was the volume of oxygen absorbed. The accuracy of the gas measurements, although not as exact as might be obtained by more elaborate apparatus, is adequate for our conclusions. Control experiments employing water, buffers, and alkali solutions without sugar showed a maximum variation of 0.2 cc. Since the oxygen absorbed was usually 10 to 30 cc., the average error was probably not greater than 2 per cent.

In those experiments in which gas measurements were made before the oxidation of the sugar was complete, small errors were introduced by the continuance of the sugar oxidation during the period of gas measurement and sampling. However, these together with the analytical errors do not materially affect the observations or the conclusions drawn from them.

Barium Peroxide—After measuring the oxygen absorbed, the entire contents of the bottle were filtered through a small filter paper and the filtrate (A) containing no detectable barium peroxide was reserved for the sugar determination. The bottle (much of the barium peroxide adheres to the side of the bottle) and the filter paper were washed twice with saturated barium hydroxide solution. After washing, the filter paper was placed in the bottle and 50 cc. of 0.5 N sulfuric acid containing 3 gm. of potassium iodide (freshly mixed) were added and allowed to stand for 30 minutes with frequent shaking. The iodine liberated was titrated with standard thiosulfate. A small blank on the sulfuric acid-potassium iodide solution was always subtracted. (The catalytic effect of molybdic acid was not known to us when these experiments were made.)

Glucose—An aliquot of the filtrate (A) was measured into a calibrated flask, diluted with a small amount of water, and the barium precipitated by the addition of a calculated amount of 2.0 N sulfuric acid. The excess acidity was neutralized with 2.0 N sodium hydroxide and adjusted with 0.1 N sulfuric acid until faintly pink to phenolphthalein. In order to provide for a few cases where an insufficient amount of sulfuric acid had been added, 5 cc. of saturated sodium bicarbonate were added routinely to precipitate any remaining barium. The bicarbonate procedure was preferable to a large excess of sulfuric acid and did not interfere with

the Somogyi modification (1926) of the Shaffer-Hartmann reagent employed for the sugar determination.

The results, plotted in Fig. 1, indicate that the barium peroxide formation, oxygen absorption, and sugar oxidation are parallel and hence dependent processes.

The same fact is shown by the data in Table I. In 0.3 M $\text{Ba}(\text{OH})_2$ at 37.5° approximately 1.7 mols of O_2 are absorbed per

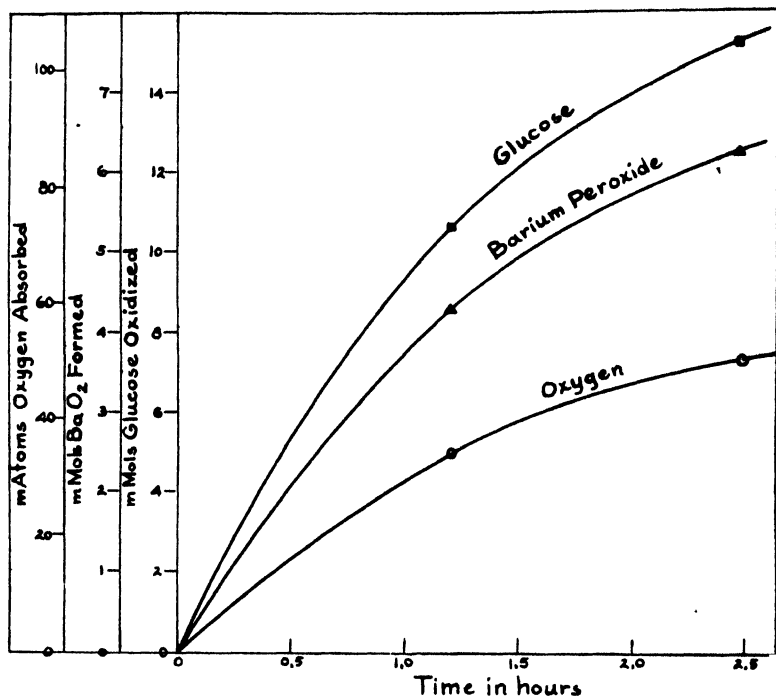


FIG. 1. Showing that oxygen absorption and BaO_2 formation parallel glucose oxidation 19 mM of glucose, 0.6 N $\text{Ba}(\text{OH})_2$; 37.5° .

mol of glucose oxidized, and about 0.5 mol of BaO_2 formed, or about 15 per cent of the total oxygen.

The velocity of the reaction is illustrated by the results for rate of loss of copper reduction capacity shown by the results in Table II. The *anaerobic* destruction of glucose is roughly about half as fast, as the rate of its oxidation at 37.5° . At 27.7° the rate of oxidation is about one-third the rate at 37.5° .

TABLE I

Oxidation of Glucose by Oxygen in 0.6 N Ba(OH)₂. Rapid Oxygenation; 37.5°

Experiment No	Time	Initial glucose	Glucose oxidised	BaO ₂ formed	BaO ₂ formed	Total oxygen absorbed	Oxygen activated
					Per mm glucose oxidised		
					mm	milli-atoms	
78	1350	19.40	18.85	10.35	0.55	3.46	15.9
79-1	972	20.30	20.02	10.95	0.55	3.44	15.9
79-2	972	20.30	20.02	10.02	0.51	3.45	14.7
82	1230	19.70	19.35	9.76	0.50	3.22	15.6
83-1*	70	18.90	10.58	4.32	0.41	3.30	12.4
83-2*	80	8.32	4.57	1.91	0.42	3.50	12.0
179	255	20.55	19.23	9.60	0.50	3.28	15.3

Fructose

37	1212	16.54	16.02	8.0	0.50		
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* The low yields of BaO₂ in Experiment 83 were due to insufficient oxygenation during the periods of gas measurement and sampling. The interruption of an experiment before the complete oxidation of the sugar always resulted in low barium peroxide yields.

TABLE II

Rate of Loss of Copper-Reducing Power of Glucose in Barium Hydroxide

	Experiment No	Time	Initial glucose	Glucose oxidized	Velocity constant (glucose values) $K = \frac{1}{t} \log_e \frac{a}{a-x}$
		min.	mm per l.	mm per l.	
Anaerobic; 0.5 N Ba(OH) ₂ ; 37.5°	172	63	20.27	5.13	0.0048
	172	85	15.14	6.26	0.0062
	172	76	8.88	3.82	0.0074
	172	79	5.06	2.10	0.0067
Oxygenated; 0.6 N Ba(OH) ₂ ; 37.5°	83.1	70	18.90	10.58	0.0117
	83.2	80	8.32	4.57	0.0099
	81.1	90	9.86	12.64	0.0113
	81.2	70	7.22	3.75	0.0122
Oxygenated; 0.6 N Ba(OH) ₂ ; 27.7°	178.4	160	20.30	8.79	0.0037

Influence of Sugar Concentration

The data given in Table III show that with an initial glucose concentration of 84 mm per liter, the oxygen absorption is rela-

TABLE III

Effect of Concentration of Sugar on BaO₂ Yields. Rapid Oxygenation; 0.6 N Ba(OH)₂; 37.5°

Experiment No.	Time	Initial glucose	Glucose oxidised	BaO ₂ formed	BaO ₂ formed	Total oxygen absorbed	Oxygen activated
					Per mm glucose oxidised		
	min.	mm per l.	mm per l.	mm per l.	mm	milli-atoms	per cent
82	750	9.80	9.36	5.45	0.58	3.61	16.1
86 .	720	28.60	28.30	13.79	0.49		
80-1	1200	41.20	40.85	14.85	0.36	3.11	11.7
80-2	1320	61.15	60.76	15.60	0.26	3.10	8.3
127-1	62	76.40	38.90	6.92	0.18		
127-2	1290	76.40	76.00	12.42	0.16		
80-3	1440	84.45	84.01	9.66	0.12	2.75	4.2

TABLE IV

Effect of Change in Normality of Ba(OH)₂ on BaO₂ Yields. Rapid Oxygenation

	Experiment No.	Ba(OH) ₂	Time	Initial glucose	Glucose oxidised	BaO ₂ formed	BaO ₂ formed	Total oxygen absorbed	Oxygen activated
							Per mm glucose oxidized		
		<i>N</i>	<i>min.</i>	<i>mm per l.</i>	<i>mm per l.</i>	<i>mm per l.</i>	<i>mm</i>	<i>milli-atoms</i>	<i>per cent</i>
37.5°	183.2	0.60	310	20.70	19.92	9.78	0.49	3.11	15.7
	183.6	0.50	310	20.70	19.81	7.64	0.39	3.00	13.0
	183.1	0.40	310	20.70	19.61	4.30	0.22	2.85	7.7
	175.2	0.36	1200	20.00	17.02	3.16	0.19	2.84	6.5
27.7°	177.3	0.60	1005	20.00	18.66	11.61	0.63	3.57	17.7
	177.2	0.50	1005	20.00	18.55	10.08	0.54	3.26	16.6
	177.1	0.40	1005	20.00	18.29	7.05	0.39	3.29	11.8

tively less and the yield of barium peroxide only one-fifth (per mm of glucose oxidized) the yield obtained with sugar concentration of 10 mm per liter. With high concentrations of sugar it is diffi-

cult to supply oxygen rapidly enough to keep pace with activation of the sugar, thus resulting in much of the primary sugar peroxide (and barium peroxide) being used in the oxidation of activated sugar with a correspondingly low yield of barium peroxide.

Influence of Alkalinity and Barium Concentration

Increasing the $\text{Ba}(\text{OH})_2$ concentration from 0.36 to 0.6 N increases the rate of oxidation, slightly increases the total oxygen absorption, and markedly increases the yield of BaO_2 . At 27° , although the rate of oxidation is much slower, both the oxygen absorbed and the fraction precipitated as BaO_2 are greater (Table IV). High alkalinity, low temperature, and low sugar concentration are, therefore, the optimum conditions for maximum peroxide deposit.

The addition of NaOH to 0.4 N $\text{Ba}(\text{OH})_2$ to make the alkalinity 0.6 N increases both the oxygen absorbed and peroxide formed, the latter being about doubled, though not so high as in 0.6 N $\text{Ba}(\text{OH})_2$. Adding BaCl_2 to 0.4 N $\text{Ba}(\text{OH})_2$ to give 0.3 M (0.6 N) Ba similarly increases both oxygen uptake and peroxide formation, though less than is caused by increase of alkalinity alone. The sum of the increases of peroxide caused by addition of BaCl_2 and of NaOH, added to the yield in 0.4 N $\text{Ba}(\text{OH})_2$ almost exactly equals the observed yield in 0.6 N $(\text{BaOH})_2$. Greater additions of BaCl_2 to 0.6 N $\text{Ba}(\text{OH})_2$ depress slightly the peroxide formation which in almost saturated BaCl_2 becomes zero. Whether this is actually due to the BaCl_2 or to traces of iron or other catalyst contaminating the salt may be questioned. Krebs (1926) found BaCl_2 , as well as various other salts, to accelerate the oxidation of sugars by air. The velocity of oxidation in presence of BaCl_2 is abnormally great for that alkalinity and temperature in spite of the lower solubility of oxygen in such solutions. The data on these points are shown in Table V.

Fraction of Oxygen Precipitated As BaO_2

In several cases close to one-half of the absorbed oxygen has been shown to be diverted to the oxidation of acceptors (Jorissen, 1897; Manchot and Herzog, 1901; Bamberger, 1900). If there were no side reactions approximately this fraction might be expected as BaO_2 in our own experiments. Instead we find at most about one-fifth. The low yield is, however, to be expected, from the fact that

TABLE
Effect of Barium Ion Concentration on BaO₂ Yields. Rapid Oxygenation

	Experi- ment No.	Ba(OH) ₂	Time	Initial glucose		Glucose oxi- dized		BaO ₂ formed		BaO ₂ formed		Total oxygen absorbed		Oxygen activated	BaCl ₂ added per 100 cc. solution
				mm per l	mm per l	mm per l	mm per l	Per mm glucose oxidized		multi-atoms					
								mm	mm						
37.5°	183-2	0.60	310	20.70	19.92	9.78	0.49	3.11		15.7	0				
	185-1*	0.40 + BaCl ₂	350	20.55	19.55	5.62	0.29	3.02		9.5	2.4				
	185-2†	0.40 + NaOH	350	20.55	19.97	8.25	0.41	3.18		13.0	0				
	183-1	0.40	310	20.70	19.61	4.30	0.22	2.85		7.7	0				
27.7°	177-3	0.60	1005	20.00	18.66	11.61	0.63	3.57		17.7	0				
	180-1	0.60	885	20.98	19.95	12.71	0.64	3.54		18.0	4.0				
	180-2	0.60	885	20.98	19.95	11.32	0.57	3.56		15.9	8.0				
	180-3	0.60	885	20.98	19.95	10.68	0.54	3.64		14.7	12.0				
	178-3	0.56	990	20.30	20.14	9.58	0.48	3.16		15.1	25.0				
	177-1	0.40	1005	20.00	18.29	7.05	0.39	3.29		11.8	0				
	178-2	0.36	990	20.30	20.14	0	0	2.83		0	98 per cent satu- rated with BaCl ₂ at 27.7°				
	178-1	0.36	160	20.30	12.85	$\text{Velocity constant} \left(\frac{1}{t} \log_e \frac{a}{a-x} \right) = 0.0067$									

Total barium ion content equivalent to 0.30 M (same as in 0.6 N Ba(OH)₂).

NaOH added to make total alkalinity 0.6 N.

TABLE
Effect of Powdered Glass and Methylene Blue on BaO₂ Yields. Rapid Oxygenation

	Experi- ment No	Ba(OH) ₂ <i>N</i>	Time <i>min.</i>	Initial glucose <i>mm per l.</i>	Glucose oxidized <i>mm per l.</i>	BaO ₂ formed <i>mm per l.</i>	BaO ₂ formed		Total oxygen absorbed		Oxygen activated <i>per cent</i>	Additional factors
							BaO ₂ formed <i>mm</i>	Per mm glucose oxidised	BaO ₂ formed <i>mm</i>	Total oxygen absorbed <i>milli-atoms</i>		
37.5°	106-1	0.60	1320	16.95	16.60	8.60	0.52					None
	106-2	0.60	1320	16.95	16.60	9.61	0.58					0.35 mm methylene blue per liter
	106-3	0.60	1320	16.95	16.60	9.59	0.58					Glass powder*
27.7°	176-1	0.50	780	49.10	46.68	18.01	0.39	2.88	13.4	Glass powder*		
	176-2	0.50	780	49.10	46.68	15.20	0.33	2.87	11.4	None		
	178-5	0.60	990	20.30	18.96	11.10	0.59	3.48	16.8	"		
	178-6	0.60	990	20.30	18.96	13.10	0.69	3.52	19.6	Glass powder*		
	181-0	0.60	365	20.73	17.43	12.78	0.73	3.55	20.6	4 gm. BaCl ₂ per 100 cc. solution; glass powder†		

* 3 gm. of purified glass powder per reaction bottle.

† 5 gm. of purified glass powder per reaction bottle.

H_2O_2 , BaO_2 , and presumably the primary sugar peroxide all oxidize the activated sugar. Some of the sugar peroxide is doubtless consumed by oxidation within the same molecule, since more than a molecule of oxygen per molecule of sugar is absorbed. It is to these side reactions, outlined earlier in this paper, that we attribute the low and variable yield of BaO_2 .

Effect of Methylene Blue and Powdered Glass

The addition of methylene blue appears to increase slightly the production of barium peroxide (Table VI). Powdered glass, which had been carefully purified to eliminate metallic impurities, also increased slightly the peroxide yield, without affecting the amount of oxygen absorbed. A possible interpretation of this fact is that the added surface of the glass aided in maintaining a more perfect oxygenation, thereby reducing to a minimum the oxidation of activated sugar by sugar peroxide and thus increasing the barium peroxide.

SUMMARY

1. An activation of molecular oxygen by the aeration of reducing sugars in alkaline solution is demonstrated by the detection of H_2O_2 with NaOH , and by the isolation of barium peroxide from $\text{Ba}(\text{OH})_2$ solutions.

2. Under optimum conditions approximately one-fifth of the oxygen absorbed appears as barium peroxide.

3. The yield of barium peroxide is decreased by increase of sugar concentration, by decrease of alkalinity, and by increase of temperature.

4. No peroxide other than H_2O_2 has been directly identified in the sugar solutions, though reasons are given for the view that the primary product is a peroxide of the sugar, H_2O_2 being formed by its reaction with water and hydroxyl ions. An outline of the hypothetical reactions is given.

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FURTHER STUDIES ON THE METABOLISM OF ESKIMOS

BY PETER HEINBECKER

(From the Departments of Surgery and Biological Chemistry, Washington University School of Medicine, St. Louis)

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It has long been considered the rule that normal persons during a period of fasting develop a ketosis. In 1928 the writer reported certain studies on the metabolism of Eskimos (1). Among other findings it was noted that although ketosis appeared with three Eskimos during a 3 day fast, the degree of ketosis was not comparable to that generally reported for other human subjects. It was not possible to decide with the evidence available whether the small degree of ketosis could be regarded as the result of continued combustion of small amounts of glycogen, thus providing a ketogenic balance as postulated in Shaffer's analysis, or was to be interpreted as indicating some adaptation for the complete combustion of fat without intervention of carbohydrate. The data on respiratory metabolism obtained in the hope of deciding between these interpretations were not deemed adequate to warrant a definite statement, though the respiratory quotients of the 3rd fast day, were 0.71 and lower, indicating little carbohydrate oxidation.

In order to repeat the observations on the degree of ketosis developed by Eskimos during a fasting period and especially to secure new and possibly more dependable data on respiratory metabolism, the author again visited the Eastern Arctic during the summer of 1930. The previous observations were made at Cape Dorset, Baffin Island (latitude 64°) on apparently full blood Eskimos living in their natural state at the time. The present report is based on studies made on apparently full blood Eskimos at Pangnirtung, Baffin Island (latitude 66°). The subjects investigated were so called "company" and "police" Eskimos who for some seasons had been in the employ of the Hudson's Bay Company and the Royal Canadian Mounted Police. This employ-

ment resulted in their securing considerable carbohydrate in their diet. This difference in dietary composition as compared with that of Eskimos living in their natural state may be of significance in the interpretation of results herein reported.

Four Eskimos were utilized for the fasting experiment. They were housed in a three room dwelling placed at our disposal by the Hudson's Bay Company agent. This dwelling also served as

TABLE I
Quantitative Urine Analyses

Subject	Day of fast	24 hr. urine volume	Reducing substances		pH
			Fermentable	Non-fermentable	
		cc.	gm	gm	
Maria	1	1247	0 070	0.660	6.2
	2	932	0 087	0.869	5.8
	3	1587	0.157	1.154	6 0
	4	1656	0 070	2 113	6.0
Simoni	1	473	0.205	0.951	6.4
	2	739	0 098	1.138	6 0
	3	798	0.178	1 441	6.3
	4	1271	0.00	1 205	6.6
Nukingklak	1	946	0 023	0.866	6.0
	2	532	0 020	0.522	6 0
	3	961	0 091	1 052	6 0
	4	1191	0 00	1.069	6 9
Imaki	1	775	0.004	0 643	5 4
	2	782	0 020	0 747	5 5
	3	569	0.002	0.663	5.3
	4	666	0.00	0 660	6.4

The urine samples gave negative tests for albumin.

the laboratory. The subjects were under constant supervision and were found most cooperative. Two of them, one a nursing mother in the late stages of pregnancy, fasted 96 hours; the other two for 115 hours from the time of last food intake. During the time 24 hour urine specimens were collected and measured. Samples of the urine, preserved with toluene, were bottled and brought to St. Louis where they were analyzed for acetone, diacetic

and hydroxybutric acids by the Shaffer-Marriott method, and for total nitrogen by the Kjeldahl method. Ammonia nitrogen was determined by the permutit method. Non-fermentable and fermentable reducing substances calculated as glucose were determined. For this, untreated urine was diluted 1:10 and the fermentable and non-fermentable reducing substances determined according to the technique of Somogyi (2) (1927), a washed yeast and a sensitive Shaffer-Hartmann reagent containing 1 gm. of KI per liter being used. The urine samples¹ were tested for protein (negative in all) and the pH determined (Table I).

On each of the first 3 days of the fast, daily basal metabolism and respiratory quotient determinations were made. The expired air was collected in 5 minute periods in a Douglas bag, after which the volume was measured by a gas meter. Samples were immediately analyzed for oxygen and carbon dioxide in a Henderson-Haldane gas burette. The subjects, in their usual clothing, rested on a bed for 30 minutes before and during each respiration period. The temperature of the improvised laboratory varied from 14–18°. Body temperature (mouth), pulse, and respiration rates were recorded. The degree of activity during the fast was well controlled. The subjects were permitted to rest in the sun outside the house but performed no work.

Ketosis during Fasting

The results as regards degree of ketosis (Table II) are in general agreement with those of our previous report. With each of the subjects ketosis appeared and increased with the duration of the fast. The ammonia excretion also rose slowly. With one exception, however, the amounts of acetone bodies excreted by the subjects were small, reaching only 0.2 to 0.8 gm. total (as hydroxybutyric acid) on the 4th fast day. These amounts are much less than is usually found after fasts of like duration. The fourth subject developed a well marked ketosis on the 3rd and 4th fast days, excreting in the urine 2.2 gm. and 7.8 gm. of total hydroxybutyric acid, respectively, amounts comparable with those commonly observed at this stage of fasting. This subject had, however, the double drain of lactation and advanced pregnancy, which

¹ All the urine analyses in St. Louis were made by Mr. E. Adler under the direction of Dr. P. A. Shaffer.

doubtless increase the depletion of glycogen reserves. The subject in our earlier experiments who showed marked ketosis on fasting was also a nursing woman, though not pregnant.

These facts appear to indicate that although Eskimos develop ketosis on fasting, which ketosis may become marked under exceptional conditions, its degree with most subjects is on the 3rd and 4th fast days much lower than commonly observed.

TABLE II
Quantitative Urine Analyses

These results are for 24 hours.

Subject	Day of fast	Urine volume	Total N	NH ₂ -N	Total acetone bodies in urine as β -hydroxybutyric acid
		cc	gm	gm	gm
Maria	1	1247	10 7	0 505	0.16
	2	932	14 9	0 758	0.30
	3	1587	15 2	0 784	0.36
	4	1656	12 5	0 830	0.22
Simon	1	473	7 15	0 466	0.10
	2	739	11 43	0 770	0.72
	3	798	13 15	1 279	2.2
	4	1271	10 92	1 880	7.8
klak	1	946	8 76	0 496	0.12
	2	532	5 66	0 313	0.10
	3	961	12 16	0 590	0.25
	4	1191	6 90	0 530	0.20
Imaki	1	775	10 88	0 374	0.10
	2	782	11 87	0 502	0.19
	3	569	10 39	0 474	0.28
	4	666	7 60	0 736	0.83

The respiratory data and nitrogen excretion should permit calculation of the ketogenic ratios of these subjects by Shaffer's factors, were it not for the fact that basal data do not indicate the total metabolism. As a rough approximation we may estimate this as at least 25 per cent above the basal. Since the determined basal energy exchange is for one subject (the lactating woman) showing most ketosis much higher on the 3rd day than the Du Bois

normal for that height, weight, and age, we give calculation for this subject based also on an assumed normal basal (figures in parentheses (Table III)). The respiratory quotients for the 3rd fast days are with all four subjects 0.70 or below, indicating no carbohydrate oxidation other than derived from protein and the glycerol of fat. It may, therefore, be supposed that only fat and protein were being metabolized. For the 4th fast days, on which respiratory data were not determined, the total metabolism is assumed to be the same as on the 3rd fast day. The calculated

TABLE III
Ketogenic Ratios for 3rd and 4th Fast Days

Total metabolism = basal plus 25 per cent.

Subject	Day of fast	Ketogenic Glucose ratio	Ketosis β -hydroxy- butyric acid excreted	Basal calories per hr.	Urine total N per 24 hrs.	Weight
			gm.		gm.	kg.
Maria	3	1.8	0.36	56	15.2	45.0
	4	2.0	0.22		12.5	
Simoni	3	2.6 (2.1)	2.20	88 (63)	13.1	62.0
	4	2.8	7.80		10.9	
Nukingklak	3	2.2	0.25	60	12.2	62.0
	4	2.9	0.20		6.9	
Imaki	3	2.2	0.28	53	10.4	52.5
	4	2.6	0.83		7.6	

The figures in parentheses are calculations based on an assumed normal basal.

ketogenic:glucose ratios (in mols) for the 3rd and 4th days are given in Table III.

The ketogenic ratios, above 2 in all but one subject, are usually accompanied by much greater ketosis than here observed. Obese subjects, however, appear to have exceptionally low ketosis. The obese subject of McClellan, Spencer, Falk, and Du Bois (3) showed little or no ketosis with ratios of 1.6 to 1.8, while four other subjects had increasing ketosis when the ketogenic ratios rose above about 1.0, becoming marked between 1.5 and 2.0. Mason (4) reports no acetonuria by ferric chloride test in obese subjects

TABLE IV
Respiratory Exchange during Fasting

Day of fast	Body temperature	Pulse rate	Respiratory rate	Cc CO ₂ consumed per min	Cc CO ₂ expired per min	R Q	Calories per hr	Calories per sq.m. body surface per hr.	Du Bois standard
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Maria; female, age 15 yrs., weight 45.1 kilos, height 146 cm.; surface area 1.34 sq.m.

	°F.								
1	98.2	75	19	221	163	0.74	62.6	47.0	43.0
2	98.0	75	22	223	169	0.76	63.6		
	98.0	74	22	234	168	0.72	66.0		
3	97.5	65	20	201	140	0.70	56.4	42.0	

Simoni; female, age 34 yrs., weight 62.5 kilos, height 155 cm.; surface area 1.59 sq.m. Nursing child and pregnant

1	98.2	70	24	274	168	0.61	73.5	46.2	36.5
2	98.0	78	23	214	159	0.74	60.7	38.2	
	98.0	78	23	241	160	0.66	64.9		
3	98.0	73	23	314	217	0.69	88.0	53.1	
	98.0	73	23	312	214	0.69	85.0		

Nukingklak; female, age 38 yrs., weight 62 kilos, height 143 cm.; surface area 1.49 sq.m.

1	98.2	65	22	191	138	0.72	53.9		36.5
	98.2	65	22	183	134	0.73	51.8	34.8	
2	97.2	52	18	237	172	0.72	57.8		
	97.2	52	18	219	161	0.74	62.1		
3	97.2	52	19	211	148	0.70	56.8	38.1	

Imaki; female, age 50 yrs., weight 52.2 kilos, height 148 cm.; surface area 1.43 sq.m.

1	97.2	59	19	163	108	0.66	43.9	30.7	36.0
	97.5	50	20	166	116	0.70	44.7		
	97.5	50	20	170	116	0.68	45.7		
2	97.2	52	17	154	105	0.68	41.4		
	97.2	52	17	159	109	0.68	42.8		
3	98.2	62	21	183	125	0.68	49.2		
	98.2	62	21	196	135	0.69	52.7	36.9	

Richards; male, age 37 yrs., weight 67 kilos, height 173 cm.; surface area 1.80 sq.m.

1	97.0	60	16	257	200	0.78	73.6	40.9	39.5
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on low carbohydrate reduction diets, corresponding to high ketogenic ratios. The very obese patients of Means, studied by Folin and Denis during repeated fasts, developed ketosis more tardily with each fast but eventually it always became marked. Whether ketosis would have become more marked on prolonging the fasts with the Eskimos it is not possible to say. With two it was greater on the 4th day than on the 3rd, and with two not. On the whole, it seems probable that the relation of ketogenic ratio to degree of ketosis does not hold with Eskimos, as apparently it does not with some other human subjects. Presumably, this means either that such subjects, like other animal species, utilize small amounts of carbohydrate more efficiently as ketolytic or antiketogenic material, or that the phenomenon of ketosis requires some other interpretation.

Respiratory Metabolism Results

The data concerning respiratory exchange are presented in Table IV. The experiments were carried out under much more advantageous conditions than those of our previous report. Daily analyses of atmospheric air were quite consistent. Their average value for the 3 days was 20.85 per cent oxygen; 0.03 per cent carbon dioxide. The pyrogallol solution did not remove more than 20.8 per cent of oxygen on any analysis. The figure 20.75 per cent was used as the oxygen content of atmospheric air in calculations of the oxygen consumed per minute by the subject. It was possible to make duplicate analyses on most days and to check the analyses made. No fault in the technique of gas analysis or in the collection of expired air could be found. Analyses of the alveolar and expired air of normal white persons gave reasonable figures. A control basal metabolism and respiratory quotient determination was carried out on a normal white male (Richards) on the 3rd day. This test was made 16 hours after the last meal. The result is a normal one.

It will be noted that the lowest values checked quite well with those given by Du Bois for normal standards. This was not found true in 1926. The results of determinations made then were higher, approximately 33 per cent on the 3rd day of fasting. A reason for the difference may perhaps be found in the different diet of the two series of subjects. In 1926 the subjects had been

living in their natural state on a purely meat diet. Their nitrogen excretion in the urine was high, average 21.90 gm. on the 1st day of the fast. The average nitrogen excretion of the subject herein reported was only 9.33 gm. on the 1st day. Such a difference is undoubtedly the result of the difference in the diet of the two sets of subjects. The higher protein metabolism of the 1926 group is believed responsible for their higher basal metabolism.

Evidence to support the above thesis is not wanting in the literature. In Krogh and Krogh's (5) observations the total metabolism of Eskimo subjects ingesting a high protein diet was determined during a 4 day period. One series of observations taken during the hours 10:10 p.m. to 6:00 a.m. when the subjects were sleeping may be considered to approximate basal conditions. The mean heat production was 1.23 calories per kilo per hour. The mean heat production calculated from the lowest basal determination of each subject of the present series was 0.967 calories per kilo per hour or 36 per cent lower than that of the Krogh and Krogh series. The basal metabolism results of the 1926 series are approximately those obtained by Krogh and Krogh during the sleeping period.

Deuel, Sandiford, Sandiford, and Boothby (6) studied the effect of high and low protein diets over an extended period on the basal metabolic rate. Subject H. J. D., showed a change in basal metabolic rate from minus 9 to minus 20 per cent on a low protein diet and subsequently on a high protein diet changed from minus 12 to plus 9 per cent. In view of such findings it seems justifiable to present the difference in dietary habits as the explanation for the difference in basal metabolic rate recorded. Krogh and Krogh (5) have demonstrated the specific dynamic action of protein in Eskimos.

The increase in metabolic rate of the pregnant lactating female is quite striking on the 3rd day. This is coincident with the appearance of a rather marked ketosis. Acidosis was found to be associated with an increase in metabolism by Benedict and Joslin (7).

The respiratory quotients are nevertheless quite surprising, being very low (0.84, 0.61, 0.72, 0.66, 0.70, and 0.67) on the 1st fast day. Although it was not possible to detect analytical errors, the consistently low quotients suggest some caution in their

acceptance. If the view be adopted that fat is transformed to carbohydrate, the low quotients might be taken as evidence. The low ketosis might also be accounted for by such a hypothesis. The writer will leave the interpretation open. Such a mechanism would seem advantageous to persons living on a purely protein and fat diet in their natural state. Other investigators, such as Higgins, Peabody, and Fitz (8), working under ideal laboratory conditions have secured quotients of similar order in persons on a protein-fat diet. Krogh and Krogh found the respiratory quotient of Eskimos on a purely fat-protein diet to be about 0.8. The average of subjects in this series on the 3rd day of fasting was 0.68.

SUMMARY

Normal Eskimo subjects were again found to develop a very mild degree of ketosis during the period of fasting.

A pregnant lactating Eskimo excreted a much larger amount of acetone bodies; less, however, than one would anticipate from a white subject under similar circumstances.

The basal metabolism of Eskimos living on a mixed diet was found similar to that of persons living on such a diet in temperate zones.

The small degree of ketosis together with the low respiratory quotients obtained during fasting is interpreted as indicating that Eskimos are able to oxidize fats more completely than ordinary persons.

Respiratory quotients below 0.7 may or may not be taken to indicate the existence of some form of intermediary metabolism such as the conversion of protein or fat to carbohydrate, depending upon the view-point of the interpreter.

Quantitative urine analyses for total nitrogen, acetone bodies, ammonia, albumin, fermentable and non-fermentable reducing substances, and acidity during fasting are recorded.

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THE EFFECT OF VARIOUS ORGANS ON THE ACETONE CONTENT OF THE BLOOD IN PHLORHIZIN AND PANCREATIC DIABETES

BY HAROLD E. HIMWICH, W. GOLDFARB, AND A. WELLER

(From the Department of Physiology, Yale University, New Haven)

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Inasmuch as the accumulation of acetone bodies in the blood of a diabetic animal may exert a toxic influence, the study of acetone metabolism has been the subject of numerous investigations. The early observations made on surviving organs indicated that the perfusion of the liver with the amino acids, leucine, phenylalanine, or tyrosine caused an increased acetone content of the perfusate (Embden, Salomon, and Schmidt, 1906; Embden and Oppenheimer, 1912; Masuda, 1912; Loeb, 1914). That the liver was the only source of acetone bodies was concluded from experiments on excised tissue which was perfused with the animal's own blood (Almagia and Embden, 1905; Embden and Kalberlah, 1906). The conversion of part of the β -hydroxybutyric acid of the perfusing fluid of the liver to acetoacetic acid was noted by Snapper and Grünbaum (1927). However, neither compound was oxidized in the liver. Acetoacetic acid injected into normal and depancreatized dogs remained longer in the blood of the depancreatized animals despite the fact that the muscles of both types of experimental animals utilized acetone with equal facility (Chaikoff and Soskin, 1928-29). The authors concluded that the liver of the depancreatized dog produced acetone bodies. In the present study of phlorhizinized and depancreatized dogs an attempt was made to determine the effects of the various organs by analyses of samples of their afferent and efferent blood for acetone bodies.

Method

The operative procedures and methods of sampling the blood entering and leaving organs have been described in other papers

from these laboratories (Himwich, Koskoff, and Nahum, 1929-30; Himwich, Chambers, Koskoff, and Nahum, 1931). The viscera investigated were liver, muscle, kidney, and the organs drained by the portal vein. Blood was drawn from the depancreatized dogs 48 or 72 hours after the operation, and from the phlorhizinized dogs after the appearance of definite amounts of acetone in the urine. 64 observations were obtained on six phlorhizinized dogs, and 76 on twelve depancreatized dogs. The urine of three depancreatized dogs collected for 24 hours was analyzed for acetone bodies. Acetone determinations of blood and urine were made by the method of Van Slyke and Fitz (1917) and the error of a single determination was ± 0.8 mg. per cent.

TABLE I
Acetone Content of Blood of Phlorhizinized and Depancreatized Dogs

	Dog No.	Hepatic vein	Portal vein	Femoral artery	Femoral vein
		mg. per cent	mg. per cent	mg. per cent	mg. per cent
Phlorhizinized dogs	1	40	43	34	40
	2	46	34	14	22
	3	38	29	32	25
Depancreatized dogs	4	13	4	8	4
	5	26	5	5	18
	6	35	17	28	25

Results

The data from six dogs are presented in Table I to illustrate the effects of the various organs on the acetone content of the blood. The afferent blood supply of the liver has been calculated from the ratio of Burton-Opitz (1911), who found that 0.3 comes from the hepatic artery, and 0.7 from the portal vein. Table II contains a summary of the number of experiments in which each of the organs either added, or made no change, or removed acetone from the blood. Differences exceeding 3 times the experimental error were taken as significant.

A consideration of Tables I and II shows that there is a considerable variation in the effects of the organs investigated on the acetone content of the blood. As a rule the liver added acetone, but the results on muscle and the organs drained by the portal

vein were not so consistent. The liver added acetone bodies to the blood twenty-seven times and removed them twice. Of the twenty-three observations on muscle in which the change was greater than 3 times the experimental error, acetone was removed in twelve instances, and liberated in eleven others. The organs draining into the portal vein absorbed acetone in twelve of the nineteen significant experiments. In five of the experiments on the phlorhizinized dogs all the organs were producing acetone bodies.

TABLE II

Summary of Effects of Muscle, Liver, and Organs Drained by Portal Vein on Blood Acetone

	Muscle			Liver			Portal organs		
	Added	No change	Removed	Added	No change	Removed	Added	No change	Removed
Phlorhizinized dogs	5	5	6	11	3	2	7	5	4
Depancreatized dogs	6	5	6	16	1	0	0	9	8
Total	11	10	12	27	4	2	7	14	12

The readings are for the number of experiments in which acetone was added to, remained unchanged, or was removed from the blood.

DISCUSSION

Though the data here reported were obtained on organs studied *in situ*, they agree, in part, with the results gained by the perfusion of surviving organs. They are also in accord with the observations of Chaikoff and Soskin (1928-29) who injected acetoacetic acid intravenously, for the liver was found to be the chief source of acetone bodies. However, the present work differs from previous investigations since some experiments showed that other organs may also produce acetone bodies. This conclusion is evident from the summary of the data in Table III. In Column 2 there is listed the number of experiments in which muscle added, made no change, or removed acetone from the blood. The effects of the other organs in each set of experiments listed in Column 2 are noted in Columns 3 to 8.

It may be seen (Table III) that the liver usually added acetone, and did so regardless of the action of the other organs, which liberated acetone bodies in only eighteen of the forty-two significant observations. The organs drained by the portal vein and muscle exhibited a similarity in reaction. In the eleven experiments in which muscle added acetone, five of the seven significant results showed that the organs drained by the portal vein were also adding that substance. Similarly, in the twelve experiments in which muscle was found to remove acetone, the organs drained by the portal vein removed acetone nine times and added it only once. Differences between the femoral artery and vein were found to be within 3 times the experimental error in ten cases; in eight

TABLE III

Correlation of Changes of Blood Acetone Produced by Muscle with Those of Liver and Organs Drained by Portal Vein

Muscle		Liver			Portal organs		
		Added	No change	Re-moved	Added	No change	Re-moved
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Added	11	10	1	0	5	4	2
No change	10	9	0	1	1	8	1
Removed	12	8	3	1	1	2	9

The readings are for the number of experiments in which acetone was added to, remained unchanged, or was removed from the blood.

of these, differences between the femoral artery and portal vein were also within 3 times the experimental error.

We have calculated that if the liver weight is equal to 2.7 per cent of the body weight (Junkersdorf, 1925) and its blood flow 84 cc. per 100 gm. of tissue per minute (Burton-Opitz, 1911), the amount of acetone that could be produced by the livers of the twelve depancreatized dogs varied from 12 to 100 gm. per day. In three of the experiments in which the urinary acetone was determined, the excretion was less than 1 gm. per day. Since the maximum possible excretion of acetone through the lungs was found by Widmark (1920) and Higgins (1920) to be less than 3 gm. per day, it is evident that the other organs must have removed the acetone from the blood. Chaikoff and Soskin (1928-29) have demon-

strated that storage could not account for the acetone thus removed, and concluded that the organs must have oxidized it. That the liver is more prone to ketosis may be explained by the observations of Hunt and Bright (1926), who found that the metabolic rate of the liver per 100 gm. of tissue was approximately 20 times that of muscle and 10 times that of the other viscera.

Woodyatt (1921) and Shaffer (1921, 1922) have shown that acetone bodies are not produced by an animal until the keto-antiketogenic ratio exceeds 1. Their results have been based on observations on the entire animal, and the criterion of ketosis has been the appearance of acetone in the urine. From the present experiments it is evident that the excretion of acetone bodies by the organism is the algebraic sum of the effects of all the organs. Only when all the organs are liberating acetone bodies are the amounts excreted through the lungs and kidneys the sum of the acetone bodies produced by the entire organism.

The femoral artery was found to have a higher acetone content than the hepatic vein in five of the thirty-three experiments. This indicates that there may be some other source of acetone bodies than the organs studied.

SUMMARY AND CONCLUSION

The acetone metabolism of organs *in situ* was studied in twelve depancreatized and six phlorhizinized dogs. The liver was found to be the chief site of acetone production (twenty-seven of twenty-nine experiments); while muscle and the organs drained by the portal vein liberated acetone bodies in eighteen experiments, and removed that substance in twenty-four others. These results indicate that the acetone excretion by the kidney and lungs is the algebraic sum of the actions of the various organs.

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DISTRIBUTION OF SUGAR IN NORMAL AND DIABETIC BLOOD, AND A COMPARISON OF THE DIRECT AND INDIRECT METHODS OF MEASURING THE CORPUSCLE SUGAR*

BY JOHN R. SPANNUTH† AND MARSCHELLE H. POWER

(From the Division of Chemistry, The Mayo Clinic and The Mayo Foundation, Rochester, Minnesota)

(Received for publication, July 27, 1931)

In recent studies of the sugar in corpuscles the indirect method of measurement, that is, calculation from the analytic values for the blood sugar, plasma sugar, and the hematocrit, has generally been employed. Difficulties in the direct analysis of packed cells have been variously attributed to incomplete protein precipitation and possible shifts in the distribution of the blood sugar *in vitro*, but principally to loss of glucose by glycolysis. Wishart (24), Macleod (12), and Somogyi (20), in considering these factors, accepted the indirect determination of the cell sugar as the method of choice. Somogyi stated that the sum of the separately determined plasma and cell sugars in defibrinated blood is, as the result of a substantial loss in the cell sugar by glycolysis, 5 to 10 mg. lower than the whole blood sugar determined immediately before centrifuging. It is his belief that defibrination or the presence of moderate amounts of mineral anticoagulants does not materially alter the distribution of reducing substances between the cells and plasma, and that the small amount of intercellular plasma left with the cells after centrifugation should have only a negligible effect on the calculated value for cell sugar. Trimble and Maddock (23), on the other hand, considered incomplete separation of the plasma

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† Fellow in Medicine, The Mayo Foundation.

as a major source of error in the direct method, rather than loss of sugar by glycolysis. With rapid packing of the cells to a minimal volume, however, fairly good agreement between the direct and indirect methods was realized. Pointing out the possibilities for error in the indirect calculation, they concluded that the direct estimation of the cell sugar is as accurate as the indirect and should always be used as a check on the latter.

During an investigation of the distribution of reducing substances between the plasma and the corpuscles with reference particularly to possible differences between normal and diabetic blood, our experience with the direct determination of corpuscle-reducing substances has not been in accord with the impression conveyed by Trimble and Maddock; this may be due to the use of a different analytic method. Under definite conditions the direct and indirect methods of analysis agreed satisfactorily; the conditions are difficult to attain as a routine, however, and we have adopted the indirect calculation of the corpuscle sugar as the more reliable procedure. The results of simultaneous determinations of corpuscle-reducing substances by the two methods, together with data on the distribution of reducing substances in normal and diabetic blood and the effect of glucose and insulin on the distribution ratios are presented in this paper. The values found for the relative concentration of fermentable reducing substances corroborate in the main the work of Somogyi, who reported the first satisfactory studies dealing with the distribution of the "true sugar" or fermentable reducing substances.

Experimental Methods

Venous blood was drawn from the arm without stasis from normal and diabetic subjects, usually in the fasting state, but occasionally 3 to 4 hours after meals. Cooled, oiled syringes were used, and one portion of the blood was measured and laked immediately, while the remainder was being transferred to ice-cooled, 15 cc. centrifuge tubes containing 2 mg. of heparin for 10 cc. of blood. The heparin contributed no measurable reducing substances. Solution of the heparin, previously deposited as a thin film by evaporation, was accomplished quickly and without undue agitation. The experiments were conducted in the laboratory, so that centrifuging could be started immediately. When this was not

possible the blood was kept in an ice bath and transported to the laboratory within a few minutes. A centrifuge with a head of 13 cm. radius and capable of 3000 revolutions a minute was used. After 3 to 5 minutes of rapid centrifuging, plasma was removed for precipitation and analysis. The tube was then further spun to constant cell volume for the hematocrit reading. The readings became practically constant after 10 minutes at 3000 revolutions. Unless the cell residue was to be used for the direct determination of reducing substances, further readings after 5 to 20 minutes more centrifuging were always taken. In several experiments low temperature during centrifuging was accomplished by surrounding the 15 cc. tubes with crushed ice in large 100 cc. centrifuge cups. For the direct determination of reducing substances in the cells the plasma was completely removed, and the cells were laked in water and precipitated with 2 volumes each of $\frac{2}{3}$ N sulfuric acid and 10 per cent sodium tungstate. The tungstic acid filtrates of whole blood were prepared according to Haden's (9) modification of the method of Folin and Wu, and of plasma according to the method of Wu (25). The filtrates represented in each case 1:10 dilutions of the original fluids.

The total and residual reducing powers of the various filtrates were estimated in duplicate by the Somogyi (18) modification of the Shaffer-Hartman method, except that potassium iodide was not incorporated in the alkaline copper reagent but added after reduction according to the suggestion of DeLong (3). This device results not only in the improved sensitivity of the reagent to the smaller quantities of reducing substances, but also in better uniformity in the extent of the reduction by small amounts of glucose as compared with that produced by the larger amounts. Thus for the particular reagent used copper reductions corresponding to 0.20, 0.40, 1, 2, 4, and 6 cc. of 0.01 N sodium thiosulfate were equivalent to 9.5, 19, 46.6, 90.5, 135, and 271.5 mg. of glucose in 100 cc. of blood; or expressed on a common basis for comparison, 1 cc. of 0.01 N solution was equivalent to 0.237, 0.237, 0.233, 0.226, 0.225, and 0.226 mg. of glucose, respectively, a maximum difference in proportionality between the low and high ranges of only about 5 per cent. 0.01 N sodium thiosulfate solution was employed for the iodometric titrations, and a 10 cc. calibrated micro burette fitted with a needle tip according to Shohl (16). This facilitated

the delivery of very small drops and the adjustment of the end-points to the disappearance of the starch blue easily to within 0.01 cc. Duplicate titrations of both blanks and unknowns almost invariably agreed to within 0.01 to 0.02 cc. of the titrating reagent, the maximum error from this source in their differences thus being 0.02 to 0.04 cc. corresponding to 1 to 2 mg. of glucose in 100 cc. of blood. The reducing power from day to day of filtrates preserved in the cold was found to be constant. Bureau of Standards glucose was used in calibrating the reducing power of a given copper solution, and all solutions were carefully rechecked at frequent intervals.

The residual reductions were determined after fermentation of the tungstic acid filtrates with washed yeast. In general the technique of Somogyi (20) which requires the use of about 1 gm. of yeast (based on the original weight of the fresh cake of compressed yeast) for 10 cc. of filtrate was followed. The wide variation in the method of fermentation employed by the recent investigators who have followed Hiller, Linder, and Van Slyke (10) and Folin and Svedberg (6), in the use of a relatively large quantity of yeast and a short fermentation period (1, 5, 14, 19), suggested the necessity of carefully rechecking the technique, with the result that the time and temperature of fermentation have been extended to 15 minutes and 35° respectively. The experimental basis for these modifications will be found in Table I which shows that although incubation for 10 minutes at 35° would ordinarily be sufficient for the fermentation of normal blood filtrates and pure glucose solutions of comparable concentration (10 mg. for each 100 cc.), yet the stronger solutions would tend to be incompletely fermented under these conditions. Since considerably longer incubation of either pure solutions or tungstic acid filtrates may be resorted to without harm, 15 minutes at 35° was adopted as a routine in order to provide a greater margin of safety in the fermentation of the higher blood sugars encountered in diabetes. These slight changes should be considered as applying only to the particular method studied, and perhaps may be valid only when used in conjunction with the other analytic methods herein described. Yeast which had stood in the refrigerator for 24 to 48 hours after its first thorough washing in the centrifuge was used, because such preparations seemed to be somewhat more efficacious

than those freshly washed, and in addition yielded more nearly perfect blanks not exceeding a value equivalent to about 1 mg. of glucose in 100 cc. of blood. In other words, the reduction of distilled water or pure glucose solution treated with yeast as in the fermentation of a blood filtrate titrated the same as water not so treated, within 0.01 to 0.02 cc. of 0.01 N thiosulfate. The uniformity with which such precision may be attained is illustrated by some of the data in Table I. In those experiments in which glucose was completely removed by the 10 or 15 minute interval, the subsequent determinations also showed the complete absence of reducing substances, agreeing among themselves usually within the limit corresponding to 1 mg. of glucose in 100 cc. of blood.

In confirmation of Somogyi's observations the fermentation of whole blood before protein precipitation, a procedure which has been used in several recent studies (1, 10, 14) always yielded a higher non-fermentable residue than did fermentation of the tungstic acid filtrate. Some comparisons of this nature are shown in Table I, the tungstic acid Filtrates a, b, and c being prepared from the same samples used in the whole blood fermentation Experiments a, b, and c. In spite of wide variation of the experimental conditions, in an attempt to find an explanation for this observation, the reason still remains obscure. Whether similar differences might be encountered with other types of blood sugar reagents is not known. Subsequent work has suggested that the glutathione content of the blood may in some way be involved, a possibility which is receiving further investigation in this laboratory. Fermentation of the tungstic acid filtrates under the conditions described gave values in good agreement with the residual reductions after complete glycolysis of the blood at room temperature.

Results

The results of experiments for the purpose of comparing the direct and indirect methods of measuring the total reducing substances in the corpuscles are recorded in Table II. The values by the direct method were, without exception, substantially lower than by the indirect. We believe, with Somogyi (20), that loss of corpuscle glucose by glycolysis, the extent of which is known to depend on time and temperature, will account in the main for the

TABLE I

Effect of Time, Temperature, and Age of Washed Yeast on Fermentation of Pure Glucose Solutions, Whole Blood, and Tungstic Acid Filtrates of Blood

	No. of experiments*	Glucose in blood or undiluted sugar solution, before fermentation	Temperature	Age of yeast	Reducing values, per 100 cc., after fermentation				
					5 min.	10 min.	15 min.	30 min.	60 min.
		mg per cent	°C	hrs	mg.	mg	mg.	mg	mg.
Glucose solutions†	2	103	25	1	17	5	2	1	
	3	105	25	24	1			0	1
	1	99	25	48	28	14	10	3	5
	1	100	25	72	11	5	0	1	
	1	100	25	96	7	5	1		1
	3	100	35	1	4	1	1	0	
	4	101	35	24			1	1	2
	4	102	35	48	4	0	1	1	1
	4	100	35	72	4	1	0	1	0
	4	105	35	96	10	4	0		
	1	204	35	1	7	7	4	4	
	3	199	35	24	7	3	2	1	0
	3	201	35	48	5	3		0	1
	3	203	35	72	13	5	3		
	2	198	35	96	13	4	2	1	
<hr/>									
Whole blood									
Experiment a.		102	25	24	20	20	17	18	18
			35		21	20	24	24	29
" b.		105	35			24	24	25	26
" c.		108	35				21		
<hr/>									
Tungstic acid filtrates									
Experiment a.		102	25	24	30	20	17	15	13
			35		15	16	13	12	12
" b.		105	35		18	18	18	17	19
" c.		108	25		25	19	20	19	19
			35		22	20	18	17	17
" d.		107	35		22	15	15	16	15

* The values represent averages when more than one experiment is indicated.

† Solutions 0.1 as strong as those indicated comparable to 1:10 blood filtrates, were used for the actual determinations.

differences found. When the corpuscles were separated by centrifuging for 30 minutes the disagreement was thus much more marked than when the separation was effected in the shorter time

TABLE II

Comparison of Direct and Indirect Methods of Determining Total Reducing Substances in the Corpuscles in Heparinized Blood

	Experiment No.	Cells	Mg. glucose per 100 cc.					Time centrifuged
			Whole blood	Plasma	Corpuscles		Difference	
					Direct	Indirect		
Normal blood		<i>per cent</i>						<i>min.</i>
	1	45	109	97	93	124	+31	30
	2	46	103	96	88	111	+23	
	3	45	91	82	76	102	+26	
	4	48	114	109	106	119	+13	
	5	47	108	103	100	114	+14	
	6	45	99	93	89	106	+17	
	7	45	105	92	90	120	+30	10
	8	48	113	106	107	121	+14	
	9	47	107	99	107	116	+9	
	10	46	105	98	107	113	+6	
	11	52	108	100	106	115	+9	
	12	48	82	70	84	95	+11	
	13	45	70	58	71	84	+13	20
	14	46	73	62	75	86	+11	
	15 a*	47	101	92	90	111	+21	
15 b†				94	111	+17		
16 a*	44	122	123	106	121	+15		
16 b†				113	121	+8		
Diabetic blood	17‡	46	221	213	199	229	+30	30
	17	49	252	254	213	249	+36	
	17	45	396	402	348	388	+40	
	17	46	306	329	268	279	+11	
	18†	44	131	126	133	137	+4	10
	18†	46	228	233	218	221	+3	
	18†	45	187	187	184	187	+3	
	18†	46	130	127	130	133	+3	

* Not cooled.

† Cooled during centrifuging.

‡ In Experiments 17 and 18 a glucose tolerance test was given.

of 10 minutes. The temperature of the centrifuge necessarily varied in the different experiments. The effect of controlling this factor is illustrated by Experiments 15 b and 16 b, in which some reduction in temperature during centrifugation was attained, a

TABLE III

Comparison of Direct and Indirect Methods of Determining the Non-Fermentable Reducing Substances in Corpuscles

	Experiment No.	Mg. glucose per 100 cc.						
		Whole blood		Plasma		Corpuscles		
		Total	Non-fermentable	Total	Non-fermentable	Total (indirect)	Non-fermentable (direct)	Non-fermentable (indirect)
Normal blood	1	111	22	102	6	121	36	38
	2	104	18	95	4	113	31	34
	3	106	17	100	4	113	29	32
	4	115	21	101	3	131	37	41
	5	98	23	94	5	101	33	38
	6	86	20	79	6	94	33	36
	7	109	17	106	4	112	25	30
	8	113	23	106	6	121	37	41
	9	108	22	100	6	115	35	34
	10	104	22	95	6	114	36	39
Diabetic blood	11	131	22	126	7	135	41	40
	12	113	17	110	8	116	29	29
	13	221	23	213	7	229	32	41
	14	252	23	254	7	249	32	40
	15	396	21	402	6	388	32	39
	16	306	21	329	6	279	32	38
	17	191	16	204	9	175	28	24
	18	243	16	252	8	231	27	27
	19	168	16	168	9	168	25	24
	20	187	22	187	7	187	40	40

definitely better recovery of reducing substances as compared with the uncooled specimens, Experiments 15 a and 16 a, was realized. In Experiment 18 more complete control of the temperature at or near 0° was accomplished by repeatedly repacking the centrifuge

tubes in ice during the separation of the cells. Under these conditions the agreement between the direct and indirect methods of analysis is satisfactory. In view of the difficulty of avoiding the effects of glycolysis in the direct method in the absence of anti-glycolytic agents, the indirect method is obviously to be preferred.

The possibilities for error in the indirect determination cannot, of course, be denied. Glycolysis has still to be considered. Heparin, which was used as the anticoagulant in order to eliminate possible shifts in the distribution of reducing substances and the large changes in the hematocrit caused by the usual amounts of dry oxalate, has no inhibiting effect on glycolysis. The drawn blood must, therefore, be cooled in an ice bath immediately and the plasma obtained for analysis after a minimum of time in the centrifuge. It must also be remembered that the calculation of the corpuscle sugar involves three other quantities, the blood sugar, the plasma sugar, and the hematocrit value. When these are within the normal range it can be shown that errors in the blood sugar affect the result about twice as much as errors in the plasma sugar, and 7 to 10 times as much as errors in the hematocrit depending on the difference between the values for the blood sugar and the plasma sugar. Particular attention should therefore be given to the accuracy of the determinations of the blood sugar and the plasma sugar. The precision of the Shaffer-Hartman method of analysis is apparently satisfactory for this purpose. Its use in the determination of the much smaller non-fermentable reducing residues in the cells, where not only the relative but the absolute error might be expected to be larger than usual, is illustrated by the data in Table III. With a few exceptions the agreement between the direct and indirect measurements of these substances is rather good. The direct values are usually a little low, suggesting that the cells as measured may still be contaminated with some plasma, which is relatively much lower in non-fermentable reducing substances than the cells themselves. The effect of such contamination on the total reducing substances in the cells by the direct method would be almost negligible since the cell sugar and plasma sugar are not widely divergent.

With the methods outlined, the distribution of reducing substances in the blood of several normal and diabetic subjects has been studied. The distribution ratios of the concentrations of

fermentable reducing substances in the corpuscles and the plasma are shown in the last column of Table IV. In the seven normal subjects studied, the ratios varied from 0.75 to 0.92, averaging 0.84. In fourteen similar experiments (not shown) carried out on six normal subjects about a year and a half later, the ratios ranged from 0.79 to 0.93, with an average of 0.86. Statistically these

TABLE IV

Distribution of Reducing Substances between Cells and Plasma

	Subject No.	Cells	Mg. glucose per 100 cc.						Ratio of cell sugar to plasma sugar	
			Blood		Plasma		Cells			
			Total	Ferment- able	Total	Ferment- able	Total	Ferment- able	Total	Ferment- able
Normal blood		<i>per cent</i>								
	1	46	106	89	100	96	113	81	1.13	0.84
	2	47	115	94	101	98	131	90	1.29	0.92
	3	48	116	102	111	108	121	96	1.09	0.89
	4	49	102	81	95	92	109	69	1.15	0.75
	5	48	86	66	79	73	94	58	1.19	0.79
	6	50	109	92	106	102	112	83	1.06	0.81
	7	48	107	93	100	97	114	89	1.14	0.92
	7	48	111	88	102	96	121	79	1.18	0.82
	7	47	107	87	99	94	115	79	1.16	0.84
7	48	104	86	95	91	113	79	1.19	0.87	
7	44	106	88	98	94	116	80	1.18	0.85	
Diabetic blood	10	46	221	199	213	206	230	190	1.08	0.92
	11	43	113	97	111	102	116	91	1.04	0.89
	12	45	131	110	126	119	137	99	1.09	0.83
	13	47	188	170	189	179	187	160	0.99	0.89
	13	46	144	127	132	124	158	131	1.20	1.06
	14	31	211	197	201	198	232	194	1.15	0.98
	15	30	355	340	368	363	325	287	0.88	0.79

means are significantly higher than the average normal ratio of 0.77 given by Somogyi (20). Since the data were obtained by similar technique, we believe the comparison is legitimate, and that it indicates the existence of a factor or factors causing on the average a constant difference in the results. These cannot be identified with certainty, but it may be suggested that our analyses of imme-

diately cooled heparinized blood may tend to be slightly higher than those of Somogyi on defibrinated blood. Further, the use of a longer fermentation period may tend to give on the average somewhat higher values for fermentable blood sugar. These tendencies, as has been pointed out, would be doubly reflected in the calculated values for corpuscle sugar.

The distribution ratios we have obtained may, however, be too high. It seems reasonable to assume provisionally that the blood glucose is evenly distributed between the corpuscles and the plasma, in view of its apparently free and unhindered diffusion into and out of the corpuscles. The ratio of the concentrations of glucose in the water of the two phases should therefore be about 0.80 (4,7). A higher value (as in the present instance) would signify a relative excess of glucose in the corpuscle water. Further investigation of the validity of the analytic results and of other variables affecting the distribution ratio will be necessary before this interesting point can be definitely settled. In the meantime it seems safe to conclude that the blood glucose is, to a close approximation at least, distributed about evenly between the corpuscles and the plasma. With this view the low value of 0.59 for the average normal distribution ratio recently reported by Folin and Svedberg (7) cannot be reconciled. Possible explanations for the low results obtained by them have been suggested by Power and Peterson (13), and by Somogyi (22).

It has been repeatedly contended that the distribution of sugar in the blood of persons with diabetes is different than in the blood of normal persons, as the result of differences in the permeability of the corpuscles to glucose. The most recent announcements to this effect are those of John (11), and of Folin and Svedberg (7), who came to apparently opposite conclusions regarding the level of the corpuscle sugar relative to the plasma sugar in the blood of diabetic and normal subjects, although agreeing that the corpuscles in both instances contain less sugar than the plasma. According to the data in Table IV none of these relationships obtains, for the distribution ratios for the fermentable reducing substances in diabetes are, with one or two exceptions, approximately the same as those found in normal blood. The number of experiments is small and we cannot, therefore, regard the results as indicating any real differences between diabetic and normal blood. More exten-

TABLE V

Effect of Glucose or Insulin on the Distribution of Reducing Substances between Cells and Plasma

Experiment No.	Blood samples	Time	Cells	Mg. glucose per 100 cc.						Ratio of cell sugar to plasma sugar	
				Blood		Plasma		Cells			
				Total	Fermentable	Total	Fermentable	Total	Fermentable	Total	Fermentable
		min.	per cent								
1	Fasting (normal)		45	104	86	95	91	114	78	1.20	0.86
	After ingestion of 100 gm. glucose	15		123	105	115	111	132	98	1.15	0.88
		30		135	117	129	125	142	107	1.10	0.86
		45		131	113	126	122	137	102	1.09	0.83
		60		124	106	118	114	131	97	1.11	0.85
		120		106	88	100	96	113	78	1.13	0.81
		180		108	90	102	98	115	80	1.13	0.81
2	Fasting (normal)		45	111	93	102	97	122	88	1.19	0.91
	After 20 units insulin intravenously	30		62	44	50	45	76	43	1.52	0.96
		60		76	58	62	57	93	59	1.50	1.03
		90		75	57	64	59	88	54	1.38	0.91
		120		74	56	62	57	88	55	1.42	0.96
		180		70	52	57	52	86	52	1.50	1.00
3	Fasting (normal)		46	107	88	99	94	116	81	1.17	0.86
	After ingestion of 100 gm. glucose (20 units insulin intravenously after 15 min. blood sample)	15		113	94	107	102	120	85	1.10	0.83
		30		154	135	152	147	156	121	1.02	0.82
		45		121	102	117	112	125	90	1.07	0.80
		60		105	86	98	93	113	78	1.15	0.84
		75		82	63	70	65	96	61	1.37	0.94
		120		70	51	58	53	84	49	1.45	0.92
		180		73	54	62	57	86	51	1.39	0.89
4	Fasting (diabetic)		43	113	97	111	102	116	91	1.04	0.89
	After ingestion of 100 gm. glucose	30		191	175	204	195	174	149	0.85	0.76
		120		243	227	252	243	231	206	0.91	0.85
		180		168	152	168	159	168	143	1.00	0.90
5	Fasting (diabetic)		45	131	110	126	119	137	99	1.09	0.83
	After ingestion of 100 gm. glucose	30		228	207	233	226	222	184	0.95	0.81
		120		187	166	187	180	187	149	1.00	0.83
		180		130	109	127	120	133	95	1.05	0.79

TABLE V—*Concluded*

Experiment No.	Blood samples	Time	Cells	Mg. glucose per 100 cc.						Ratio of cell sugar to plasma sugar	
				Blood		Plasma		Cells			
				Total	Fermentable	Total	Fermentable	Total	Fermentable	Total	Fermentable
6	Fasting (diabetic)	min.	per cent								
	After ingestion of 100 gm. glucose	30	46	221	199	213	206	230	190	1.08	0.92
		120		252	230	254	247	249	210	0.98	0.85
		180		396	374	402	395	389	350	0.97	0.88
7	Fasting (hyperinsulinism)		45	306	284	329	322	279	240	0.85	0.75
	After ingestion of 100 gm. glucose	30		64	41	50	44	82	37	1.64	0.84
		60		64	42	48	43	82	41	1.71	0.95
		120		69	47	57	51	84	42	1.47	0.82
				64	45	50	45	84	45	1.68	1.00

sive data in support of this position are available in the work of Somogyi (21), together with a detailed consideration of the consequences of neglecting the non-sugar reducing fractions in blood and plasma in the investigation of this question.

That the corpuscles of diabetic and normal blood are not essentially different in the respects mentioned also seems evident from a study of the effects on the distribution ratio of experimentally induced changes in the blood sugar. After the ingestion of 100 gm. of glucose by the normal subject the differences in plasma glucose and blood glucose are well maintained during the usual rise and fall in the blood sugar curve; accordingly no significant shifts in the distribution ratios occur (Experiments 1 and 3, Table V, last column). Apparently neither the rate of increase of the plasma glucose, nor its subsequent rate of decrease is sufficient appreciably to exceed the rate of diffusion of the glucose into or out of the corpuscles, respectively. Similarly in diabetes the distribution ratios do not significantly change in spite of the much greater fluctuations in the blood sugar level after 100 gm. of glucose (Experiments 4, 5, and 6, Table V). The possibility in such experiments of the rate of increase of the plasma glucose exceeding its rate of diffusion into the corpuscles, with a consequent depres-

sion of the distribution ratio, might be anticipated. Although the number of experiments is too small to prove the point, this condition seems to be realized in Experiment 4, in which the ratio determined on the rapidly rising blood sugar curve is definitely depressed. The change is only temporary, however, and need not necessarily signify decreased permeability of diabetic corpuscles to glucose.

In contrast to the difficulty of detecting more than a temporary change in the distribution of glucose when the blood sugar is increased, is the definite shift of the ratios toward high values when the blood sugar is decreased under the influence of insulin. This effect in the normal subject is shown in Experiment 2, Table V; the differences in plasma glucose and blood glucose become practically nil, and the distribution ratios consequently approach the value 1.00. The later determinations in Experiment 3 show the same tendency, as does also Experiment 7, which represents a glucose tolerance test in a case of "hyperinsulinism." On account of the possibilities for a greater error in the analytic determinations and hence also in the calculated concentrations for corpuscle glucose and in the distribution ratios at the low blood sugar levels reached with insulin, the interpretation of these results must be approached with caution. Suffice it to say that provided errors are not obscuring the real situation, the changes in the distribution of fermentable reducing substances seem to be in accord with Cori's (2) view that the function of insulin is to accelerate the utilization of glucose by the tissues.

The effect of insulin on the distribution of glucose in the blood of diabetic persons has not been investigated by us, as during the course of the work the papers of Shope (17) and of Trimble and Maddock (23) covering this point were published. Their data show that the administration of insulin to persons with diabetes does not result in marked shifts in the distribution of reducing substances in the blood. Foshay (8) has suggested that insulin reactions might occur in diabetes as the result of the development of a "cytoglucopenia," without a correspondingly low blood sugar. In view of the opposite effect of insulin on the relative concentration of glucose in the corpuscles in normal subjects demonstrated above, and the published evidence just cited, these contentions of Foshay as well as similar ones of Rona and Sperling (15) must rest apparently on incomplete or erroneous data.

SUMMARY

The reducing substances in the corpuscles of heparinized blood have been determined simultaneously by the direct and indirect methods of analysis. Comparable results were obtained only when careful precautions to prevent glycolysis were observed. The indirect calculation of the corpuscle sugar is believed to be the more reliable procedure, especially when antiglycolytic agents are not used.

Under the conditions of the experiments the average ratio of the concentrations of fermentable reducing substances in the corpuscles and the plasma of normal persons was found to be 0.84. The ingestion of 100 gm. of glucose by a normal subject results in practically no change in the distribution ratio, either on the ascending or descending parts of the sugar tolerance curve. The intravenous injection of insulin, however, results in material increases in the distribution ratio. This indicates a tendency toward a relative excess of glucose in the corpuscles, probably due in part to the accelerated rate of removal of glucose from the plasma during the action of the insulin.

When 100 gm. of glucose are taken by patients with diabetes the rate of increase of the glucose in the plasma may exceed the rate of diffusion into the corpuscles, with a consequent temporary downward shift in the distribution ratio and hence in the concentration of glucose in the corpuscles relative to the plasma. Other than this, the data at hand indicate that the distribution of fermentable reducing substances in the blood of persons with diabetes, and the changes in distribution incident to the ingestion of glucose, are not different from those found in normal persons. There is thus no evidence that the permeability of the corpuscles to glucose, or the content of glucose in the corpuscles relative to the plasma, is any different in diabetic than in normal persons.

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THE COMBINED SUGAR OF TUNGSTIC ACID FILTRATES OF BLOOD

BY FREDERICK H. SCHARLES AND EDWARD S. WEST

(From the Departments of Medicine and Biological Chemistry, Washington University School of Medicine, St. Louis)

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Tompsett, in a recent paper (1), has presented data which he interprets as indicating the presence of an easily hydrolyzable sugar complex in tungstic acid filtrates of blood. He implies that this "bound" sugar is blood glucose though presenting no evidence that it is either glucose or sugar.

His conclusions were based upon observations made with a modified Shaffer-Hartmann method (2) which he claims is practically unaffected by the non-sugar reducing substances of blood.

His evidence, in brief, is as follows: He found that $\text{HgSO}_4\text{-BaCO}_3$ filtrates of blood gave lower reduction values than tungstic acid filtrates, these being similar to those reported by West, Scharles, and Peterson (3). He found also that treatment, as described by the above workers, of fresh tungstic acid filtrates with solid $\text{HgSO}_4\text{-BaCO}_3$ removed a part of the reducing material. However, if the tungstic acid filtrates were allowed to stand at room temperature for several days (preserved with benzoic acid), then $\text{HgSO}_4\text{-BaCO}_3$ treatment caused no change in reduction. He then concludes that since his method of analysis is not appreciably affected by non-sugar reducing substances of blood, the difference between the fresh tungstate and the mercury filtrates is due to bound sugar which is precipitated by mercury but not by tungstic acid, and that mercury treatment of old tungstate filtrates causes no change in reduction because the bound sugar has been hydrolyzed to products which cannot be removed by mercury. This reasoning presumes that his method gives identical results for the sugar of blood irrespective of whether that sugar is entirely free or partly in the bound condition.

In view of the fact that Tompsett's rather surprising conclusions seemed to be based upon inadequate observations obtained by a questionable technique, the writers have repeated some of his experiments and carried out others. Aside from checking up on his method of analysis and repeating his essential experiments, with the addition of fermentation controls, a study of the fermentable and non-fermentable fractions in tungstic acid filtrates as influenced by acid hydrolysis has been made.

Tompsett's procedure of analysis was as follows: He separated the Shaffer-Hartmann reagent into a copper-tartrate-carbonate solution (Solution 1) and an iodide-iodate-oxalate solution (Solution 2). 10 cc. of blood filtrate were heated 15 minutes with 10 cc. of Solution 1, the reduced copper centrifuged down, washed with H_2O , 10 cc. of Solution 2 added, followed by 10 cc. of $N H_2SO_4$. The iodine was titrated with 0.0125 N thiosulfate. Tompsett precipitated tungstic acid filtrates with solid $HgSO_4 \cdot BaCO_3$ as recommended by West, Scharles, and Peterson but removed mercury with copper instead of zinc or H_2S .

This method of analysis is unnecessarily tedious and cumbersome, but with considerable practice the writers were able to obtain satisfactory values with pure glucose solutions. The essential error in Tompsett's method of analysis seems to be the use of copper to remove mercury from the filtrates. The writers at an early stage of their work on mercury precipitation found that unless much more acid is used than is added by Tompsett, mercury is only incompletely taken out by copper. However, the use of more acid, bringing about complete removal of mercury, is no better because such filtrates still give quite erratic reduction values. The reason for this is not entirely clear, although it may be that Cu in contact with Cu^{++} produces enough Cu^+ to vitiate the results.

Table I gives data obtained from a repetition of Tompsett's experiments. Tungstic acid filtrates of human blood were saturated with benzoic acid and allowed to stand 3 and 7 days. Reducing substances were determined by using Tompsett's modified Shaffer-Hartmann reagent and procedure both before and after precipitation of the filtrates with solid $HgSO_4 \cdot BaCO_3$. In addition to removing Hg^{++} with Cu according to Tompsett's procedure, we also removed it with H_2S . Our results with Tompsett's

method (with Cu) entirely fail to show the increased reducing values which he claims result from standing. The values are erratic and inconclusive. When Hg^{++} was removed by H_2S , however, it will be seen that the reduction remained constant, showing no significant change in either the original or final tungstate or $\text{HgSO}_4\text{-BaCO}_3$ tungstate filtrates.

In order better to test the hypothesis we repeated Tompsett's work, using the sensitive Shaffer-Hartmann reagent containing

TABLE I

Effect of Standing on Reducing Substances of Tungstic Acid Filtrates of Blood Saturated with Benzoic Acid

Tompsett modification of Shaffer-Hartmann reagent and procedure and $\text{HgSO}_4\text{-BaCO}_3$ precipitation used.

Time	Tungstic acid filtrate (a)	HgSO ₄ -BaCO ₃ precipitation of (a)		Reducing non-sugar (H ₂ S) (a - b)	Reducing non-sugar (Cu) (a - c)
		H ₂ S used for removal of Hg (b)	Cu used for removal of Hg (c)		
	mg per cent	mg per cent	mg. per cent	mg per cent	mg. per cent
Original.....	120	96	106	24	14
7 days later.	120	95	67	25	53
Original.....	123	97	93	26	30
3 days later.....	124	94	87	30	37

1 gm. of KI per liter, and in addition fermented the mercury filtrates according to the method of Somogyi (4).¹ Table II shows that the reducing values of the tungstic acid filtrates, before and after treatment with $\text{HgSO}_4\text{-BaCO}_3$, remained constant after standing 7 days. Also the reducing material present in the mercury filtrates was completely fermentable by yeast within the limits of error of the method.²

¹ Mercury removes sufficient benzoic acid to permit rapid fermentation.

² It is evident that blood contains some non-fermentable simple sugar since a considerable quantity is excreted in the urine (West, Peterson, and Lange, unpublished data). The amount present is so small, however, that it cannot be determined in mercury filtrates by present methods of analysis.

A study of the reducing substances of acid-hydrolyzed tungstate filtrates was made. A large amount of filtrate was prepared from

TABLE II

Reducing Substances in Tungstic Acid Filtrates of Human Blood Saturated with Benzoic Acid and Permitted to Stand at Room Temperature
Sensitive Shaffer-Hartmann reagent used.

Time	Tungstic acid filtrate (a)	HgSO ₄ -BaCO ₃ filtrate of (a) (b)	Reducing non-sugar (a - b)	Yeast fermentation of (b)
	mg. per cent	mg. per cent	mg. per cent	mg. per cent*
Original.	105	77	28	
7 days later.	109	81	28	0
Original.	148	125	23	
7 days later.	150	122	28	1(?)
Original.	106	83	23	
7 days later.	108	82	26	0
Original.	200	174	26	
7 days later.	202	174	28	0
Original.	101	79	22	
7 days later.	100	80	20	
Original.	90	73	17	
7 days later.	91	70	21	0
Original.	284	262	22	
7 days later.	58	40	18	0†

* There generally remained the reducing equivalent of 2 to 3 mg. of glucose in the fermented HgSO₄-BaCO₃ filtrates. It was found that yeast, the supernatant liquid of which gave no blank, when fermenting pure glucose solutions of approximately the concentration of the mercury filtrates, produced about this amount of reducing material. The figures in the column have been corrected by subtracting this value.

† No preservative used.

mixed human blood, and fermentable and non-fermentable reducing substances were determined both before and after hydrolysis with 0.5 N H₂SO₄ for varying lengths of time.

Fig. 1 shows total fermentable and non-fermentable reducing substances plotted against time of hydrolysis. The fermentable, or true, blood sugar remained constant after 20 hours hydrolysis, while the reducing values of the non-fermentable substances increased to a maximum at about 5 hours and declined a little below the original after 20 hours.³ It is also of interest to note that the sugar found in the $\text{HgSO}_4\text{-BaCO}_3$ filtrates at all stages of hydrolysis corresponded with the fermentable sugar.

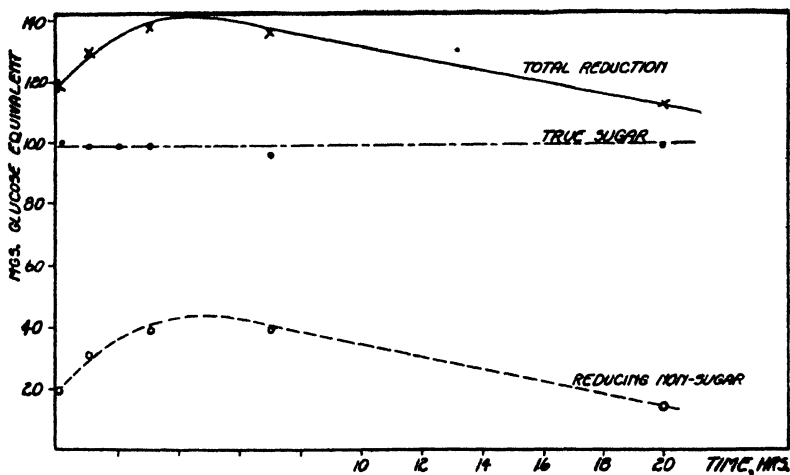


FIG. 1. Total, fermentable, and non-fermentable reducing substances plotted against time of hydrolysis.

The fact that mercury removed completely not only the non-fermentable reducing material in the unhydrolyzed filtrate but also the reducing products formed by hydrolysis, indicates that there is not only no bound glucose in tungstic acid filtrates but also that no detectable part of the reducing non-fermentable material is *sugar of any kind*.⁴ Apparently, however, there must be some non-carbohydrate complex present which upon hydrolysis yields unstable reducing substances. This cannot be glutathione

³ Everett, Shoemaker, and Sheppard (5) also noted, with some tungstic acid filtrates, that acid hydrolysis caused increased reduction followed by a decrease.

⁴ See foot-note 2.

which has been shown to constitute a considerable fraction of the reducing non-fermentable material of tungstic acid filtrates (6) since Somogyi⁵ has found the reducing power of glutathione to be very little affected by acid hydrolysis.

SUMMARY

1. Tompsett's conclusions that tungstic acid filtrates of blood contain an easily hydrolyzable sugar complex have not been confirmed.

2. The reducing non-fermentable fraction of tungstic acid filtrates increases upon acid hydrolysis, attains a maximum, and falls below the original value within 20 hours. This variation cannot be due to glutathione but indicates the presence of some material which upon hydrolysis yields an unstable reducing substance (or substances). The fermentable sugar remains unchanged.

3. The non-fermentable fraction of tungstic acid filtrates apparently contains no detectable sugar of any kind because $\text{HgSO}_4\text{-BaCO}_3$ precipitation of hydrolyzed filtrates removes this reducing substance completely at all stages of hydrolysis and gives values identical with those for fermentable sugar.

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⁵ Personal communication from Dr. Somogyi.

THE EFFECT OF LARGE DOSES OF IRRADIATED ERGOSTEROL UPON NITROGEN, CALCIUM, AND PHOSPHORUS METABOLISM IN RATS

BY RUTH KERN, MARY F. MONTGOMERY, AND EUGENE U. STILL

WITH THE TECHNICAL ASSISTANCE OF JOHN BOERSMA AND
VICTOR HUNKLE

(From the Department of Physiology of the University of Chicago, Chicago)

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The production of toxic phenomena in animals by the feeding of large doses of irradiated ergosterol has been reported so many times as to require no further confirmation. However, certain aspects of the process remain obscure. The studies reported in this paper were undertaken to determine the effect of varying doses of irradiated ergosterol upon the rate of growth and upon the nitrogen, calcium, and phosphorus metabolism of young rats upon a normal diet. Since the experiments were begun, in the fall of 1929, balance studies of calcium and phosphorus metabolism with rats on high dosages of irradiated ergosterol have been published by Light, Miller, and Frey (1929), Brown and Shohl (1930), and Watchorn (1930), and studies upon infants and dogs by Hottinger (1929). We also wished to observe whether the solvent in which the ergosterol is irradiated bears any relationship to the toxicity of the irradiated product, or whether toxicity runs parallel with the vitamin content of the sample, regardless of the mode of irradiation. Dixon and Hoyle (1928), using ergosterol irradiated in oil, could not obtain toxic effects with doses as low as the ones used by some German workers and pointed out that the method of irradiation, particularly the solvent used, might be the factor underlying the development of toxicity. Windaus (1930) was of the belief that the antirachitic activity and the toxicity were the properties of the same substance. However, Holtz and Schreiber (1930) later published from his laboratory evidence supporting the view that the toxic factor was a separate entity. They state that oxida-

tion or warming with sodium alcoholate of toxic irradiated ergosterol destroyed the antirachitic property and not the toxicity.

Shultz and Meyer (1930) favor this view-point. They believe the great discrepancy in the levels of vitamin dosage at which different workers obtain toxic effects is evidence against the toxic effects being simply a matter of vitamin dosage. On the other hand, Harris and Moore (1929) believe that an excessive dosage of vitamin D may, of itself, produce a specific condition of hypervitaminosis aside from the as yet undemonstrated action of any toxic products. They found that ergosterol activated in oil or in alcohol, when fed at the same vitamin D levels, produced toxic effects of like severity and that ergosterol irradiated dry was also toxic. Toxicity disappeared from irradiated ergosterol when the vitamin content was destroyed by over-irradiation. Non-irradiated and resinized ergosterol were non-toxic. They believe that they have shown a definite relationship between vitamin content and toxicity. Harris and Innes (1931) have reported the effect of varying amounts of calcium and phosphorus on the development of vitamin D hypervitaminosis. They have made balance studies; and in the main our findings are in agreement with theirs. We believe that the experiments to be described in this paper throw some additional light upon the question of ergosterol toxicity, and especially the effects upon metabolism.

Methods

1. Ergosterol Irradiation

The ergosterol used in these experiments was all obtained from a single sample prepared by Mead Johnson and Company (99 per cent pure). One portion was non-irradiated, and separate portions were irradiated dry, in dry freshly distilled ether solution, and in redistilled absolute ethyl alcohol solution. All irradiation was done at a distance of 35 cm. for 35 minutes with a Cooper Hewitt mercury arc lamp. No filter was used. The solvents were removed *in vacuo*.

2. Assay of Ergosterol

The three types of irradiated ergosterol were assayed by determining the smallest daily dose which would effect a 2+ cure, in 8

days, as determined by the line test. The results of the assay are shown in Table I.

3. Collection of Urine and Feces

Rats were used as experimental animals. They were placed in individual metabolism cages of the type described by Still and Koch (1928) with the modification that no filter paper was placed over the funnel. We found that by using a food container with a small opening, scattering was reduced to a minimum. In the case of individual rats which scattered considerable quantities of food, this food was recovered from the urine by filtration, and was dried, weighed, and subtracted from the amount eaten. In these cases allowance was also made for the soluble constituents of the food which passed into the urine. The daily food intake was re-

TABLE I
Assay of Irradiated Ergosterol

Sample	Manner of irradiation	Daily curative dose	
		mg.	per mg.
D	Dry	0.0005	2000
E	In ether	0.0005	2000
A	" alcohol	0.001	1000

corded. The urine and feces were collected and analyzed for nitrogen, calcium, and phosphorus in periods of 4 days.

4. Methods of Analysis

Nitrogen was determined in the urine and feces after digestion with H_2SO_4 by the macro-Kjeldahl procedure. Fecal calcium and phosphorus were determined upon aliquots of the wet ashed material by the methods of McCrudden and of Fiske and Subbarow respectively. Urinary calcium was determined by McCrudden's method either upon ashed urine or directly upon the filtered specimen. Urinary phosphorus was determined by the method of Fiske and Subbarow on H_2SO_4 -digested samples.

Methods of Bone Analysis—Each bone was freed from most of the attached muscle and was at once immersed in acetone. At the end of a few hours the remaining soft tissues were scraped away.

The bone was broken into several pieces, which were enclosed in a small filter paper and were extracted in a Soxhlet extractor with ether for 2 hours, then with absolute alcohol for another 2 hours, and again for a final 2 hours with ether. After being dried *in vacuo*, the bone was ground to 100 mesh, and the fibrous material was removed. The powder was again extracted with alcohol for 1 hour and with ether for 1 hour. Samples prepared in this manner were stored *in vacuo* until the analyses were made. The analytical procedures used were those described by Shear and Kramer (1928), with the exception that phosphorus was determined by the method of Fiske and Subbarow.

TABLE II
Experiments I, II, and III

Group No	Rat No.	Ergosterol, non-irradiated	Ergosterol, irradiated
		<i>mg.</i>	<i>mg.</i>
1	1, 2	9	0
2	3, 4	8	1
3	5, 6	6	3
4	7, 8	4	5
5	9, 10	2	7
6	11, 12	0	9

5. Diet

In all of the experiments reported in this paper, the diet consisted of:

	<i>per cent</i>
Casein.....	20
Starch.....	71
Dried yeast.....	5
Complete salt mixture (Osborne and Mendel, 1913).....	4

10 gm. of butter were added to each 90 gm. of the dry mixture, and (except in the case of the first experiment) a sufficient quantity of the ration was made up to last for a complete experiment of the series. The food was analyzed for nitrogen, calcium, and phosphorus. Throughout the series these values varied as follows: nitrogen from 2.6 to 2.8 per cent, calcium from 0.48 to 0.58 per cent, and phosphorus from 0.58 to 0.68 per cent.

6. Plan of Experiments

Experiments I, II, and III—Experiments on twelve young rats of approximately the same weight (divided into six groups of a male and a female each) were run consecutively. The rats were permitted to eat as much of the ration as they would and, in addition, a small piece of lettuce was given every 4th day. Ergosterol mixed with a portion of the ration was fed daily at varying levels (Table II). Each experiment lasted for eight balance periods of 4 days each and was identical with the other two except for the type of ergosterol fed. The dry irradiated ergosterol was used in Experiment I; the ergosterol irradiated in ether in Experiment II; and the ergosterol irradiated in alcohol in Experiment III.

Experiment III-B—At the end of Experiment III the rats which had received ergosterol irradiated in alcohol for 32 days were maintained for 24 days longer under identical conditions with the exception of the daily ergosterol dosage. This was raised to 30 mg. in Group 2, to 45 mg. in Group 3, and to 60 mg. in Group 4. The dosage was not altered in the case of Group 1 (controls) and Groups 5 and 6. The latter were kept upon the same dosages of 7 and 9 mg. of the irradiated ergosterol respectively because we wished to determine whether or not these dosages would prove toxic if fed for a longer time.

Experiment IV—The number and sex of the rats used were the same and the plan of the experiment was similar to that of the others with the following exceptions. Instead of being permitted to eat at will, each individual rat received the same total quantity of food in the experimental period of 40 days. All three types of ergosterol (irradiated in alcohol, irradiated in ether, and dry irradiated) were fed in relatively high dosages as indicated in Table III. One femur and tibia of every rat were analyzed for Ca, P, and CO₂. The thigh muscles and one kidney of the rats in Experiments III-B and IV were analyzed for calcium. Microscopic sections of heart, aorta, and kidney were also made in the case of the latter animals.

Results

Experiments I, II, and III—The rats in these experiments showed no deviations from the normal which could be interpreted as evidences of toxicity. The growth of all was at the normal rate.

TABLE III
Total Balance Data on Rats in Experiment IV Receiving Large Doses of Ergosterol Irradiated in Alcohol, in Ether, or Dry over a Period of 40 Days

Group No.	Rat No. and sex	Ergosterol dosage		Body weight		Nitrogen			Calcium			Phosphorus			Balance								
		Non-irradiated		Original	Final	Food intake			Food			Feces			Urine			N		Ca		P	
		mg.				gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent		
1	1 ♂	25 A	25	60	180	358.7	10264	508	5468	1722	279	163	2439	232	1627	4288	41.7	1280	74.3	580	23.8		
	2 ♀	25 A	25	62	164	359.0	10270	562	6123	1724	214	134	2441	264	1570	3586	34.9	1376	79.8	607	24.8		
2	3 ♂	50 A	0	60	176	359.0	10270	426	5120	1724	98	275	2441	152	1658	4724	46.0	1351	78.4	631	25.8		
	4 ♀	50 A	0	62	148	351.4	10052	564	5175	1687	159	302	2390	265	1614	4314	42.9	1227	72.8	511	21.4		
3	5 ♂	25 E	25	58	174	359.0	10270	654	5767	1724	440	81	2441	325	1577	3849	37.5	1203	69.8	539	22.1		
	6 ♀	25 E	25	52	158	359.0	10270	665	5955	1724	475	94	2441	405	1557	3650	35.6	1155	67.2	479	19.6		
4	7 ♂	50 E	0	64	178	359.0	10270	645	5862	1724	519	130	2441	394	1628	3763	36.6	1075	62.4	419	16.9		
	8 ♀	50 E	0	64	156	359.0	10270	550	5740	1724	245	140	2441	276	1635	3980	38.7	1339	77.7	530	21.7		
5	9 ♂	25 D	25	60	178	359.0	10270	555	5464	1807	405	75	2441	336	1584	4251	41.4	1327	73.4	521	21.3		
	10 ♀	25 D	25	52	156	359.0	10270	557	5821	1724	400	75	2441	350	1561	3892	37.9	1250	72.5	530	21.7		
6	11 ♂	50 D	0	58	172	359.0	10270	603	5544	1724	396	136	2441	350	1567	4123	40.2	1192	69.2	524	21.4		
	12 ♀	50 D	0	60	152	356.2	10190	610	5756	1710	263	123	2423	277	1531	3824	37.5	1324	77.4	616	25.5		

* In this column A means irradiated in alcohol; E, irradiated in ether; and D, irradiated dry.

There were no changes in the N, Ca, and P balance nor in the proportion of those elements excreted in the urine and feces. The levels of retention for the 32 day periods were:

Experi- ment No.	N per cent	Ca per cent	P per cent
I	26.2-43.4	50.5-63.6	30.1-37.4
II	27.3-41.2	40.5-65.2	16.8-29.1
III	27.6-43.7	55.2-67.9	26.6-36.0

Experiment III-B—Groups 1, 5, and 6, which remained upon the same dosage of ergosterol, all continued to grow at the expected rate. The percentage nitrogen retention was lower, as one would expect it to be in the more adult rat. The excretion of phosphorus in the feces remained at the same general level, while the values for urinary phosphorus climbed slowly and steadily. The percentage of phosphorus retained during these 24 days was 9.5 to 15.9 per cent, that is, approximately one-third to one-half as great as the retention had been during the preceding 32 days. In Group 1 (controls, no irradiated ergosterol) there was no significant change in the urinary or fecal calcium. The percentage retention of calcium was slightly less than it had been (39.8 to 46.9 per cent). The rats in Groups 5 and 6 showed an increased urinary calcium in the last two periods and the two females also showed a somewhat diminished fecal calcium. The calcium balance remained positive at a lower level than before, but retention was greater than in the case of the controls (49.9 to 54.9 per cent).

Of the rats on the high doses of irradiated ergosterol the male receiving 30 mg. showed a good gain in weight and the expected level of nitrogen retention. The female receiving 45 mg. gained 8 gm. during the 24 days. All the others ate less and lost weight, and showed a decreased retention of nitrogen. In Table IV we present the complete balance data from Experiments III-A and III-B, for male Rat 7, which received first a low dose and later a very high dose of ergosterol irradiated in alcohol. The first part of Table IV is quite characteristic for all rats in Experiments I, II, and III. Rat 8 (female, receiving 60 gm.) which lost a total of 14 gm. of body weight, had a negative nitrogen balance for three periods, and at the end of the experiment had a positive balance of only 70 mg. of nitrogen. Whatever changes were observed in the nitrogen metabolism could be entirely explained upon the

Experiment No.	Date	Weight	Food	Nitrogen			Calcium			Phosphorus			Balance					
				Food	Feces	Urine	Food	Feces	Urine	Food	Feces	Urine	Nitrogen	Calcium	Phosphorus			
		gm.	gm.	mg	mg	mg.	mg	mg.	mg.	mg.	mg.	per cent	mg	per cent	mg	per cent		
III-A	Jan. 14	70																
	" 17	80	28.65	802	70	259	143	13.8	2.9	172	14.7	63.9	473	59.0	127.0	88.8	93.6	54.3
	" 21	96	34.40	963	66	396	172	23.3	5.5	206	20.5	92.1	501	52.0	143.2	83.2	83.4	40.4
	" 25	112	39.40	1103	82	554	197	41.0	8.8	236	29.9	108.1	467	42.4	147.2	74.7	96.0	41.5
	" 29	122	37.40	1047	88	540	187	75.7	6.3	224	53.4	104.1	419	40.0	105.0	56.1	66.5	29.6
	Feb. 2	138	36.40	1019	70	547	182	48.8	9.4	218	35.0	108.7	402	39.4	123.8	68.0	74.3	34.0
	" 6	152	37.40	1047	90	581	187	55.9	9.1	224	39.8	110.5	376	35.9	122.0	65.2	73.7	32.9
	" 10	166	37.40	1047	91	546	187	62.3	8.8	224	40.4	110.5	410	39.1	115.9	61.9	73.1	32.6
	" 14	172	36.40	1019	96	625	182	72.9	8.5	218	50.0	109.3	298	29.2	100.6	54.1	58.7	26.9
	" 18	184	37.40	1047	100	679	187	101.1	8.9	224	65.8	110.5	268	25.6	77.0	41.1	47.7	21.3
III-B	Feb. 22	192	33.00	924.0	57	577	165.0	42.5	26.5	198.0	25.9	104.7	+290	+31.3	+96.0	58.2	+67.4	34.0
	" 26	186	25.35	709.8	95	516	126.8	25.1	17.8	152.1	14.3	125.0	+98.8	+13.9	+26.9	21.2	+12.8	8.4
	Mar. 2	182	24.65	668.0	57	603	128.2	9.7	51.6	147.9	11.9	136.0	+8.0	+1.2	+66.9	52.2	0	0
	" 6	180	21.80	590.8	50	544	113.4	9.1	55.2	130.8	10.8	128.6	-3.2	-0.5	+49.1	43.3	-8.6	-6.6
	" 10	178	26.45	716.8	40	587	137.5	9.1	55.8	158.7	10.7	137.8	+89.7	+12.5	+72.6	52.8	+10.2	6.4
	" 14	184	29.20	791.3	27	570	151.8	10.2	56.1	175.2	6.2	133.4	+194.3	+24.5	+85.5	56.3	+35.6	20.3

In Experiment III-A this rat received 4 mg. of ergosterol irradiated in alcohol and 5 mg. of non-irradiated ergosterol daily; in Experiment III-B it received 60 mg. of ergosterol irradiated in alcohol daily.

basis of decreased food intake. The animal developed a distaste for food from the 5th to the 16th day of the experiment, and on certain days ate nothing at all. It has been observed repeatedly that rats can distinguish irradiated ergosterol in the food. We thought that the rat might be starving itself to avoid eating the ergosterol. We therefore fed the ergosterol by force separate from the food for a few days at the height of the period of anorexia. An improved food intake was observed at once, and although the amount eaten never returned to normal the rat slowly gained back 10 gm. after a loss of 26 gm. We also had to resort to forced feedings of ergosterol in the case of Rat 7 (male, receiving 60 mg.). This rat, too, showed an improved appetite and gain of weight after a time of anorexia, loss of weight, and negative nitrogen balance. Because of the constancy of the urinary nitrogen we do not feel that the renal function was greatly impaired.

The fecal and urinary phosphorus for the three groups under discussion was lower on an average than in the case of the controls, and negative balances were observed a number of times during the experiment. Phosphorus retention varied from 10.2 to 19.5 per cent for the entire period of the experiment.

From the very first period all rats showed a great increase in the excretion of calcium in the urine and a much diminished calcium content of the feces. A negative balance was noted in the case of Rat 8 (female, receiving 60 mg.) for one 4 day period only, during which time the urinary calcium was 74 mg. Rat 7 excreted 74.8 mg. of calcium during the same period but the balance did not become negative. After this tremendous peak both these rats continued to excrete calcium at a constant level. Rat 8 excreted 36.3 to 38.9 mg. per period, and Rat 7, 51.6 to 56.1 mg. per period. As we have stated, the continued normal excretion of nitrogen is evidence against the production of any extensive kidney damage due to the large excretion of calcium.

In spite of the greatly increased urinary output of calcium, the total percentage of calcium retained was 44.0 to 56.5 per cent except in the case Rat 8, whose calcium retention was 34.5 per cent.

It seems very likely that negative calcium balance does not develop as long as the food intake remains adequate.

The autopsy findings and tissue analyses are discussed together with those of rats in Experiment IV.

Experiment IV—The total balance data for the 40 days of this experiment are given in Table III. No difference was observed

TABLE V

Mg. of Calcium and Phosphorus Excreted by the Male Rats of Experiment IV

During the first three periods all rats consumed, in a given period, within 12 mg., the same quantity of calcium. During the rest of the experiment the differences were never greater than 5 mg.

Date	Calcium		Phosphorus		Calcium		Phosphorus		Calcium		Phosphorus	
	Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces
	Rat 1 fed 25 mg. ergosterol A daily				Rat 5 fed 25 mg. ergosterol E daily				Rat 9 fed 25 mg. ergosterol D daily			
Apr. 20	5.2	12.1	103.3	12.7	4.3	14.2	98.8	17.9	5.2	13.0	109.7	15.4
" 24	7.3	24.5	137.5	14.6	6.2	20.4	131.3	18.8	4.6	23.8	158.8	23.1
" 28	9.2	21.9	161.8	18.9	5.6	30.0	157.2	28.5	4.9	29.5	160.6	27.4
May 2	18.3	20.8	189.8	21.1	7.5	50.4	178.8	17.9	12.3	39.3	166.1	34.5
" 6	19.9	22.6	170.4	19.5	7.9	46.8	171.3	30.0	5.7	37.6	163.6	29.4
" 10	11.5	31.6	145.1	24.7	7.3	39.5	143.6	26.7	4.7	40.6	144.9	30.0
" 14	14.4	46.4	182.9	39.8	8.9	55.4	172.4	42.1	6.5	53.0	169.4	42.5
" 18	22.0	37.6	190.3	31.9	10.9	58.2	203.4	45.9	10.4	50.2	176.1	41.9
" 22	24.8	51.3	185.7	34.9	12.4	65.1	174.0	52.7	12.4	70.3	172.3	54.0
" 26	30.8	9.9	160.7	13.6	9.8	60.2	146.6	44.2	8.4	48.0	142.5	37.9
	Rat 3 fed 50 mg. ergosterol A daily				Rat 7 fed 50 mg. ergosterol E daily				Rat 11 fed 50 mg. ergosterol D daily			
Apr. 20	10.2	16.2	105.1	14.0	6.8	22.9	119.4	22.9	6.1	14.1	105.0	16.7
" 24	22.3	19.8	150.4	18.5	10.7	32.6	163.5	20.5	9.7	19.6	140.2	17.9
" 28	24.4	10.9	158.4	9.7	12.3	31.3	163.0	23.4	12.7	33.9	158.4	34.8
May 2	29.0	14.0	177.3	25.5	14.0	46.6	169.8	36.8	5.9	31.3	159.6	32.1
" 6	25.9	11.8	176.0	17.0	12.3	55.0	161.3	36.4	13.6	38.9	159.3	33.5
" 10	22.3	3.0	153.5	5.3	8.9	55.2	147.7	39.2	9.7	34.9	145.1	30.8
" 14	24.7	6.4	174.3	15.9	13.9	66.2	174.3	50.0	14.5	48.2	172.5	37.7
" 18	34.5	7.9	197.5	18.4	18.4	73.8	189.8	61.1	21.0	58.1	198.4	50.3
" 22	40.5	3.2	192.6	16.0	20.5	72.2	180.7	57.9	25.0	68.0	171.7	57.5
" 26	41.6	4.6	172.8	11.9	12.3	62.1	158.3	45.5	17.4	49.1	156.4	38.8

Ergosterol A, E, and D are ergosterol irradiated in alcohol, in ether, and dry, respectively.

in the growth of these rats as compared with those in Experiments I, II, and III, except in a single instance. Rat 4, female, grew

satisfactorily until the 34th day of the experiment and then lost 14 gm. This occurred in spite of the fact that all of the rats ate nearly identical quantities of food during the experiment. Table V shows the calcium and phosphorus excreted by the male rats during each period of the experiment.

Nitrogen—Nitrogen retention was satisfactory throughout the experiment and averaged a little higher in the males than in the females. The amount of nitrogen excreted in the urine in any given period by any one animal was roughly proportional to the quantity of food eaten in that period. There was no suggestion of altered digestion, absorption, or retention. The amount of nitrogen retained diminished both in mg. and per cent as the animals approached the adult state.

Calcium—Table IV (Experiment III-A) shows the proportion of the ingested calcium which was normally excreted in the urine and feces. In our relatively large series of balance studies on growing or young adult rats the urinary calcium varies from 4 to 8 mg. per 4 day period and the fecal calcium contains from one-third to one-half or more of the ingested calcium, depending on the age of the animal. All of the animals in Experiment IV, receiving the ergosterol irradiated in alcohol, excreted a much larger proportion of calcium in the urine (Tables III and V), and in Group 2 the urinary calcium actually became higher than the fecal calcium. This reversal became evident during the second period and continued until the end of the experiment. So marked was the change in the rats receiving this dose of ergosterol that in certain periods calcium almost disappeared from the feces. Values for fecal calcium for some periods fell to 0.8 to 2.5 mg. per day, whereas normal rats of the same weight, consuming the same quantity of the diet, would excrete from 15 to 25 mg. of calcium in the feces. In view of these extremely low values for fecal calcium we believe we are justified in concluding that we are dealing with an increased absorption of calcium from the intestinal tract and probably a diminished intestinal excretion as well.

Of the rats on ergosterol irradiated in ether and ergosterol irradiated dry (Tables III and V) the ones receiving 25 mg. of irradiated ergosterol showed a small increase in the urinary calcium and a small decrease in the fecal calcium. The ones receiving 50 mg. of irradiated ergosterol showed a more marked increase in

urinary calcium, and with the exception of Rat 7, male, a marked diminution of the fecal calcium. These changes began to be evident anywhere from the second to sixth period of the experiment.

In spite of the increased excretion of calcium in the urine, the entire group of rats retained a higher percentage of the calcium ingested than the apparently normal rats of Experiments I, II, and III. A part, at least, of this calcium was deposited in the tissues such as the heart, aorta, and kidney, as was shown by microscopic sections of those organs.

It should be pointed out that whereas some workers have reported negative calcium and phosphorus balances in rats receiving large doses of irradiated ergosterol, our rats (Experiment IV) remained throughout the experiment in a high positive calcium balance. We believe that the maintenance of the food intake is of great importance in that respect and by such maintenance our animals have been able to deposit (abnormally) calcium, whereas, on a lower intake they would have been in negative balance.

If one accepts the profound disturbance in calcium metabolism as a criterion, there is no doubt that the rats receiving ergosterol irradiated in alcohol were much more toxic than the rats receiving either ergosterol irradiated in ether or irradiated dry. This statement is even more significant when one recalls that ergosterol irradiated in alcohol was only one-half as potent in the cure of experimental rickets as are ergosterol irradiated in ether and ergosterol irradiated dry. From these experiments we are forced to conclude that, whatever its relation to vitamin D content, toxicity appears to be to a certain extent related to the method of irradiation. However, we do not believe the method of irradiation to be the only or even the main factor involved. In some unreported experiments we produced death or extreme emaciation, in young rats, in 21 days, by feeding 5 mg. daily of an ergosterol preparation irradiated in alcohol which contained 10,000 curative doses (daily) per mg. Although the food intake of the animals was not controlled and fasting may well have been a factor, we feel the effect of the vitamin content must not be underestimated.

Phosphorus—The rats receiving high dosages of irradiated ergosterol excreted less phosphorus in the feces and more phosphorus in the urine than the apparently normal animals in Experiments I, II, and III. This was especially true for those animals

receiving ergosterol irradiated in alcohol. Compare Table V with Table IV. Due to the fact that the increase in urinary phosphorus was greater than the decrease in fecal phosphorus, the net result was a lower phosphorus balance.

Autopsy Findings

Experiment III-B—No gross changes were observed in any of the organs of the rats of Experiments III-B and IV except in the kidneys. Stone formation was observed in a single instance (male

TABLE VI

Per Cent of Calcium Found in Ash of Muscle and Kidney Taken from Rats Receiving Large Doses of Irradiated Ergosterol

Rat No and sex	Calcium in ash			
	Kidney		Muscle	
	Experiment III-B	Experiment IV	Experiment III-B	Experiment IV
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1 ♂	1.83	0.41	4.06	2.21
2 ♀	12.50	18.65	2.93	3.15
3 ♂	1.52	9.84	3.03	1.98
4 ♀	24.81	19.54	0.96	0.77
5 ♂	2.11	17.26	0.94	1.31
6 ♀	26.34	22.01	1.09	0.75
7 ♂	5.31	2.33	1.39	0.86
8 ♀	21.98	9.27	1.53	0.87
9 ♂	1.18	2.18	1.20	1.29
10 ♀	21.09	33.78	1.00	0.80
11 ♂	1.05	2.66	0.99	2.27
12 ♀	14.69	36.70	0.86	0.97

Rat 3, Experiment III-B). However, many of the rats showed a shiny, whitish zone between cortex and medulla, and under the dissecting microscope spicules of calcium could be lifted out of the tissues in this area. This change was invariably more evident in the female rat of a pair receiving the same dose of ergosterol. On examination of the rats used in Experiment III-B such depositions were noted in the case of all the rats upon the higher dosages of ergosterol (30, 45, and 60 mg.) and these rats with the exception of Rat 3 showed small intercellular calcium deposits. There was, however, no evidence of calcium accumulation in the collecting

tubules. From our gross observations, our experience in Experiment II, and from the analysis of the tissues we are inclined to believe that such intratubular concretions existed but were washed away in the preparation of the tissues for microscopic examination.

Experiment IV—Large calcium deposits were found within the lumina of the collecting tubules in the case of Rats 2 to 6, 8, 10, and 12; none in the case of Rats 1, 7, 9, and 11. Intercellular deposits were much less frequent than in rats of Experiment III-B, showing kidney changes. It is evident that the females (even numbers) showed the deposition more often than the males. This relationship is brought out in a striking way in Table VI where we have tabulated the results of the analysis of kidney tissue for calcium. In every instance in Experiments III-B and IV the calcium content of the kidney ash was higher in the female of the group than in the corresponding male. This held for twelve groups of rats on dosages of irradiated ergosterol up to 60 mg. daily. We are at a loss to explain this apparent sex relationship.

Heart

Experiment III-B—2+ deposits were found in the muscles of all rats receiving 30, 45, or 60 mg. of irradiated ergosterol daily.

Experiment IV—Small calcium deposits were present in the case of Rats 2, 4, 5, 8 to 10, and 11, and were observed chiefly beneath the pericardium.

Aorta

Experiment III-B—Small calcium deposits were present in the aortæ of the rats receiving 45 and 60 mg. of irradiated ergosterol.

Experiment IV—Small calcium deposits occurred in Rats 1 to 4, 6, 9, 10, and more extensive ones in Rats 8 and 11.

Analysis of Muscle and Bone

The analyses of the thigh muscles of these animals showed no abnormalities of calcium content which could be associated with the conditions under which the animals were kept (see Table VI). Table VII records the analyses of the bones of all animals in the entire group of experiments for Ca, P, and CO₂. No changes in the calcium content of the bone occurred under the conditions of the experiments. If any unusual amount of calcium was deposited or removed from the bone as a result of the high dosage of ergo-

TABLE VII
Analysis of the Leg Bones of All Rats Used in These Experiments

Rat No.	Experiment I, ergosterol D				Experiment II, ergosterol E				Experiment III, ergosterol A				Experiment IV, ergosterols A, E, and D			
	Ca	P	CO ₂	Ca:P	Ca	P	CO ₂	Ca:P	Ca	P	CO ₂	Ca:P	Ca	P	CO ₂	Ca:P
	per cent	per cent	per cent		per cent	per cent	per cent		per cent	per cent	per cent		per cent	per cent	per cent	
1	20.75	10.63	3.60	1.95	21.40	10.75	2.81	1.98	20.65	10.67	2.86	1.94	22.77	11.26	2.83	2.02
2	21.34	10.87	3.23	1.96	21.56	10.90	2.81	1.98	21.66	10.80	2.90	2.01	21.72	11.18	2.89	1.94
3	20.05	10.50	3.30	1.95	21.08	10.71	2.68	1.97	21.65	10.83	2.70	2.00	22.31	11.40	2.75	1.96
4	21.13	10.53	3.35	2.01	21.64	10.73	2.81	2.02	21.83	10.55	2.48	2.07	22.35	11.39	3.28	1.96
5	21.72	10.84	3.43	2.01	22.70	11.07	2.31	2.05	21.68	10.84	2.90	2.00	21.87	11.15	2.81	1.96
6	20.92	10.73	3.39	1.95	22.33	10.86	2.76	2.06	21.60	10.96	2.43	1.97	22.38	11.38	2.81	1.97
7	20.76	10.76	3.04	1.94	22.40	11.19	2.85	2.00	21.77	10.77	2.65	2.02	21.54	10.79	2.93	2.00
8	22.05	10.88	3.16	2.03	21.80	11.21	2.58	1.94	21.83	10.65	2.75	2.05	21.48	10.84	2.90	1.98
9	21.66	10.74	3.37	2.02	22.57	11.35	2.71	1.99	21.93	10.67	2.94	2.06	21.12	10.49	3.06	2.01
10	21.73	10.86	3.40	2.00	21.42	10.60	2.87	2.02	21.65	10.90	3.18	1.99	21.37	10.83	3.06	1.97
11	21.64	10.53	3.20	2.06	21.57	10.60	2.79	2.03	21.74	10.71	3.49	2.03	21.28	10.61	2.80	2.00
12	21.35	10.59	3.15	2.02	21.72	10.65	2.64	2.04	21.74	10.84	3.35	2.01	21.44	10.88	3.22	1.97

Ergosterols A, E, and D are ergosterol irradiated in alcohol, in ether, and dry, respectively.

sterol, there remained bone of entirely normal composition with respect to the Ca:P:CO₂ content.

SUMMARY AND CONCLUSIONS

1. In growing rats receiving large daily doses of irradiated ergosterol there was found macroscopic and microscopic evidence of the accumulation of much calcium in the kidneys. Chemical analyses of those organs revealed the deposition to be much greater in the cases of the females than in the males.

2. Irradiated ergosterol in large doses increases the absorption of calcium from the intestine. The excretion of calcium in the feces decreases and the excretion in the urine is greatly increased.

3. To a lesser degree there is also a diminished fecal elimination of phosphorus.

4. Ergosterol irradiated in alcohol produces greater disturbances in the calcium metabolism of growing rats than either ergosterol irradiated in ether or irradiated dry, on the basis both of actual weight of ergosterol fed and of the vitamin D content of the samples. We therefore believe that the medium in which the ergosterol is irradiated plays an important part in the development of toxicity.

5. The nitrogen excreted in the urine seemed to have been entirely within the normal range, being affected mainly by the level of food intake.

6. Whatever changes may occur in the way of deposition or absorption of bone in rats fed large amounts of irradiated ergosterol, analyses of the leg bones of these animals show normal contents of calcium, phosphorus, and CO₂.

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STUDIES ON THE TOXICITY OF GOSSYPOL

I. THE RESPONSE OF RATS TO GOSSYPOL ADMINISTRATION DURING AVITAMINOSIS*

BY WILLIS D. GALLUP

(From the Department of Agricultural Chemistry Research, Oklahoma Agricultural Experiment Station, Stillwater)

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INTRODUCTION

Gossypol is the toxic principle of cotton seed. When given *per os* to rabbits and cats, it produces diarrhea and such disorders of more serious consequence as paralysis of the voluntary muscles and nerve degeneration. Edema of the lungs and effusion into the serous cavities are of common occurrence (1). Similar results with cardiac involvements may follow intravenous and intraperitoneal injections of the substance (1, 2). Postmortem examination of a dog which died from daily ingestion of gossypol showed excess abdominal fluid, congestion of all the splanchnic organs, and hemorrhagic intestines (3). When gossypol is incorporated in the food of rats, the animals soon show a dislike for the food, develop diarrhea, lose weight in proportion to the amount of gossypol in the diet (4), and in some instances die in a state which resembles suffocation (5). In the cases of acute poisoning of rats, produced by intraperitoneal injections of gossypol, Clark (6) states that postmortem examination has shown congested lungs, hemorrhagic condition of all the viscera, and cardiac dilatation. When death was delayed following such injections there was intestinal impaction. Fish showed signs of suffocation in water to which 1 part per 100,000 of gossypol had been added (2). Experiments *in vitro* indicate that gossypol lowers the oxygen-carrying capacity of the blood by inhibiting the liberation of oxygen from hemoglobin (2). The effect of pure gossypol upon large animals has not been studied.

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When cotton seeds or cottonseed meal are used as experimental material to determine their relative toxicities and the physiological action of the gossypol contained therein, pathological conditions similar to those mentioned above have been noted in many animals. Postmortem examination of dogs fed upon cottonseed meal revealed hyperemic condition of the intestinal canals, engorgement of the mesenteric vessels, hemorrhagic intestines, dilated heart, and congested kidneys (3). Rabbits, guinea pigs, pigeons, and albino mice which died from eating cottonseed kernels and meal developed congestion of the liver, kidneys, and lungs previous to death. Intestinal injuries were also noted (7). The extent of the injuries produced through the feeding of these products varies considerably with different animals and with different preparations of the material. This fact brought out in the early studies of Withers and Carruth (8) and Mendel and his associates (7, 9) has led to much confusion regarding the merits of cottonseed meal as a food for live stock.¹

Cottonseed meal is decidedly less toxic than raw cottonseed kernels, the toxicity of which is directly related to their gossypol content. However, in several of the experiments noted above (2, 3, 7) cottonseed meal was shown to be capable of producing in small animals physiological disturbances and pathological changes which were similar to if not identical with those produced by feeding pure gossypol. The opinion that cottonseed meal injury is analogous to gossypol poisoning is based upon these findings. In those instances in which cottonseed meal has been fed in liberal quantities and without apparent harm to such experimental animals as albino rats or to larger animals as sheep, horses, and cows, the seemingly logical conclusion has been drawn that the meal is not toxic to them. This conclusion is supported in part by the well known fact that the gossypol in the meal is much less active physiologically than is the pure substance. Reference to numer-

¹ During the past, cottonseed meal and the products made therefrom have not found a prominent place in the human diet. Because of its content of vitamin G, the meal has quite recently been suggested as a food for people in districts where pellagra is prevalent. However, sufficient experimental work has not been done to warrant such a recommendation in view of the possible toxicity of the meal to human beings.

ous cases of injury reputed to be due to cottonseed meal is found in several of the papers mentioned (7, 9).²

One of the most vivid descriptions of the condition of cattle which have been fed upon excessive cottonseed diets is contained in a paper read before the Missouri Valley Veterinary Association, in 1899, by Dr. F. C. McCurdy (10). In view of the recent support given to the idea that cottonseed meal injury in cattle is more likely due to a deficiency than to the presence of a toxic substance in the meal, this paper is of particular interest. Quoting from McCurdy's paper (p. 560) in which a description is given of a herd of small southern cattle,

"many seemed partially blind, and in a few there was a complete loss of sight. At first glance it was supposed that they were suffering with contagious ophthalmia. As they were being driven it was noticed that they were very nervous, weak, and exhausted. They moved with an uncertain, staggering gait. Respiration seemed hurried and difficult, and there was a frequent 'scouring' discharge from the bowels.

On reaching the pens they showed a condition of nervous depression, remaining in one position, either recumbent or standing with the head down, with trembling of the limbs and without ruminating. The owner said that their poor condition was caused quite recently by his mistake of feeding too much cottonseed."

Several of the herd were slaughtered and condemned.

"The remainder of the herd were allowed to rest for five days and they were fed on hay. In that time they again commenced to ruminate, the symptoms of weakness and nervous excitement or depression had disappeared. There remained only the lesions of the eyes—a permanent opacity of the cornea, varying from a small ulcer to a bulging staphyloma, discharging pus.

Post mortem examination of the carcasses when made in the abattoir disclosed no pathological changes in the viscera. The liver, kidneys, and spleen were apparently normal in size, color, and consistency . . .

On inquiry it was learned that many cattle were seen in the stockyards, very weak and poor in condition, and these cattle have an opacity of the cornea, and often a swelling of the legs. The history of these animals is always the same. While en route, or in confinement, they have been fed too much cottonseed meal."

Huffman and Moore (11) and Halverson and Sherwood (12) have recently reported the results of extensive feeding experi-

² See especially Wells, C. A., and Ewing, P. V., *Georgia Agric. Exp. Station Bull.* 119 (1916).

ments with cottonseed meal from which they conclude that the meal may be fed in liberal quantities without producing toxic effects in dairy cows providing it is supplemented with good quality roughage such as hay and alfalfa. However, when the source of roughage is confined to beet pulp, cottonseed hulls, or wheat straw, the animals go "off feed" and display symptoms typical of cottonseed meal injury. The latter investigators state (p. 156).

"Typical symptoms of 'cottonseed meal poisoning' have been obtained in cows by feeding peanut meal, linseed meal or soybean meal with a mineral mixture and a poor roughage, thus confirming the conclusion that this condition is not due to the presence of a toxic substance in cottonseed meal. Evidence has been presented which shows that the failure of the cows on these rations was due primarily to a deficiency of vitamin A."

Such results as these, which are not entirely new, are presented in support of what has been termed the "deficiency theory" of cottonseed meal injury.

Vitamins As Possible Factors in Cottonseed Meal Injury

An examination of the diets used in the recent studies of gossypol poisoning in small animals does not lead one to suggest that the diets were lacking in any of the known vitamins. There remains the possibility that the presence of gossypol in the diet either added or carried in the form of cottonseed meal raises the animals' requirements for certain vitamins or other dietary essentials which might otherwise be present in adequate amounts. The experiments reported in this present paper relate to this possibility.

Several years ago Rommel and Vedder (13) drew the conclusion that cottonseed meal poisoning in pigs was analogous if not identical with beriberi. Halverson and Sherwood ((12) p. 40) report that the condition of cows on cottonseed meal and hull diets was greatly improved by administering vitamin B concentrates such as yeast and wheat embryo. In unpublished experiments at this station, cod liver oil has been found to be of value in promoting the recovery of dairy cattle suffering from cottonseed meal injury. An interesting report in this connection has been made by Robison (14) who states, "Even when fed at a 20 per cent level in a ration containing 7.9 per cent tankage, ordinary untreated cottonseed meal caused no losses." (This statement refers to pig feeding in-

vestigations, with use of ordinary and autoclaved cottonseed meal. Autoclaving reduces the toxicity of cottonseed meal by its effect on the gossypol (5, 15.) Robison found, however, that when untreated cottonseed meal was used to balance corn for pigs in dry lot, twenty-two out of thirty-seven pigs died, and since yellow corn, minerals, and alfalfa were fed he concludes that the injurious effect was not likely due to a deficiency of minerals or of vitamins A or D. Evidence in favor of the view-point that gossypol was responsible for the death of these animals lies in the fact that autoclaving the cottonseed meal for $\frac{1}{2}$ hour reduced the mortality from nine to one out of seventeen. Extending the time of this treatment reduced the number of deaths from six to none out of ten. The favorable effect of tankage in the rations lead Robison to believe that a part of the trouble with cottonseed meal was due to its proteins being incomplete or unbalanced. In recent publications (16) the author has pointed out that in cottonseed meal studies the use of various supplements in the diet which have a specific effect upon gossypol may lead to inconsistent results. To investigate this possibility further and perhaps shed some light upon the manner in which gossypol acts, the present study was undertaken.

EXPERIMENTAL

Albino rats were used in this study because their vitamin requirements are quite well known and they can be handled in large numbers. Furthermore, the minimum lethal toxic dose of gossypol, when injected intraperitoneally in oil solution, has been fairly well established for these animals and found to lie between 20 and 30 mg. per kilo of body weight. For the determination of the toxic effects of gossypol upon rats under a chosen dietary regimen, three practical methods of administering the substance are available; *viz.*, *per os*, as a constituent of the food or given by stomach tube, and intraperitoneal injections. All three methods were tried and the relative merits of each discussed in the text. In all of these experiments the rats were kept in screen bottom cages without access to feces. They were weighed every 3 or 4 days and records kept of the food intake.

Gossypol, prepared from its acetate salt according to the method of Carruth (17) and Schwartze and Alsberg (18), was dissolved in pure refined cottonseed oil and used in the intraperitoneal injection.

tions. Solutions of 0.50 per cent, which were made up fresh for each series of injections, were used in most instances. It was found that the gossypol would dissolve in the oil most readily if first dissolved in a few drops of ether and allowed to form in a thin layer on the inside of the flask as the ether evaporated. The oil was then added a little at a time and the gossypol brought into solution by shaking and only slight warming.

An ether extract of cotton seeds was used as a source of gossypol in the experiments in which the gossypol was either added to the food or given to the animals by stomach tube. The ether was evaporated from these extracts *in vacuo* and fresh extracts made each week in a battery of Soxhlet extractors. Gossypol determinations were made according to methods previously described (15) in which the gossypol is precipitated and weighed as its aniline salt. This method, although the best available, gives consistently low results which contribute a small error in calculating the amount of gossypol which each animal received.

When gossypol was administered by stomach tube the animals were placed under light ether anesthesia. The mouth was held open with the tongue forward by means of a small pair of forceps and the stomach tube, a No. 8 F urethral catheter inserted the proper length. A hypodermic needle was attached to the end of the tube and various amounts of the extract, usually less than 2 cc., representing known amounts of gossypol, were administered in this way with the aid of a syringe. During the short space of time which it took to insert the tube and discharge the solution the animals came out from under the anesthesia and displayed no immediate signs of injury as a result of this treatment. Several experiments carried out in this manner with pure cottonseed oil resulted in no apparent injury to control animals.

Experiments with Rats on Low Vitamin B³ Diets

Only a few experiments were conducted with animals on low vitamin B diets because of the unlikely possibility of this vitamin ever being a limiting factor in the ordinary use of cottonseed meal as food. Young rats taken from our regular stock at weaning age were maintained on a synthetic vitamin B-free diet until their stores of vitamin B had been depleted as shown

* Vitamin B complex.

by loss in body weight. This required about 15 days. The diet was composed of dextrin 58, acid-washed casein 18, Crisco 15, salts 3.5,⁴ agar 2, and cod liver oil 3.5. They were then divided into two groups and supplied with graded amounts of yeast supplement of predetermined vitamin B potency for a period of 18 days. One group, designated as the low vitamin B group in Table I, made an average daily gain of 1 gm. The other group, which served as a control, made an average daily gain of 2.5 to 3.0 gm. At the end of this period intraperitoneal injections of gossypol were made. The results are summarized in Table I.

In those instances in which the animals lived for some time following the injections their food intake during the first 2 days was negligible but gradually increased. During this time they were allowed a stock diet composed of natural foodstuffs and containing adequate amounts of vitamin B.

The data presented in Table I do not relate vitamin B deficiency in rats with an increased susceptibility to gossypol poisoning. In fact, the greater number of deaths occurred among the animals receiving adequate amounts of this vitamin although this might be explained in part by the larger size of these animals (1). Most of the animals were killed at the end of 8 days and autopsies performed. The usual conditions found were intestinal inflammation, adhesions, and considerable edematous fluid in the peritoneal cavity. This fluid was not always hemorrhagic. In several instances the liver was engorged. The heart and lungs appeared normal in all but one instance in which case the left lung was hyperemic. One of the animals in Experiment 1 and two in Experiment 4 lived for more than 20 days following the injections. Although suffering from edema they appeared quite active and maintained a normal appetite until a few days before death. Approximately 25 cc. of sterile hemorrhagic fluid were drained from each of these rats during this time.

Experiments with Rats on Low Vitamin A Diets

Intraperitoneal Injections of Gossypol—In these experiments rats were maintained upon vitamin A-free diets until their stores of this vitamin had been partially or completely depleted. Two diets

⁴ McCollum's Salt Mixture 185 (McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918)).

were employed; one was made up of dextrin 56, casein (extracted with hot alcohol) 18, Crisco 15, yeast 8, and salts 3,⁴ and the other was McCollum's rolled oat diet composed of rolled oats 40, alcohol-extracted casein 5, dextrin 52.5, NaCl 1, and CaCO₃ 1.5. Control

TABLE I

Toxicity of Gossypol (Injected Intraperitoneally in Oil Solution) to Rats on Low Vitamin Diets

Experiment No.	No. of rats used	Weight	Gossypol per kilo body weight	Character of diet	Fatalities								No survived	Duration of experiment
					1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day		
Low vitamin B and control groups														
		gm	mg.											days
1	6	56- 90	25	Low vitamin B	0	0	0	0	0	0	0	0	6	8
2	5	85-120	25	Control	0	2	0	0	0	0	0	2	3	8
3	5	75- 85	38	Low vitamin B	0	0	1	0	0	0	0	1	4	8
4	6	80-115	38	Control	0	2	0	2	0	0	0	4	2	8
5	4	50- 65	50	Low vitamin B	1	1	0	0	0	0	0	2	2	16
6	8	80-110	50	Control	1	3	0	4				8	0	4
Low vitamin A and control groups														
7	7	150-175	25	Low vitamin A	0	0	2	0	0	0	0	2	5	8
8	4	100-140	25	“ “ “	0	0	0	1	0	0	2	3	1	8
9	8	105-190	25	Control	0	0	0	2	0	0	0	2	6	8
10	2	80	38	Low vitamin A	0	0	0	0	1	1		2	0	6
11	6	110-150	38	“ “ “	3	3						6	0	2
12	5	90-102	38	“ “ “	2	0	1	1	0	0	0	4	1	8
13	5	112-145	38	Control	0	1	2	0	0	1	0	4	1	8
Low vitamin D and control groups														
14	13	72-130	25	Low vitamin D	0	2	1	1	1	2	2	10	3	8
15	5	115-140	25	Control	0	0	1	0	1	0	1	3	2	8
16	11	68-120	38	Low vitamin D	5	3	0	2	1	0	0	11	0	5
17	5	115-150	38	Control	0	1	1	1	1	0	1	5	0	7

animals received the same diets, with the exception that commercial casein was used, plus 3 per cent of cod liver oil.

Although the exact stage of vitamin depletion was never known, partial depletion was used to describe that stage at which the animals on low vitamin A diets failed to make appreciable gains in

weight for a period of 10 days but showed no positive signs of xerophthalmia. Relatively large animals which had been reared on adequate diets containing cod liver oil during an early period of life were used for this. They were fed on the low vitamin A diets for at least 4 weeks previous to the injection of gossypol. Xerophthalmia with loss of weight marked complete vitamin A depletion.

Intraperitoneal injections were made as described and the animals continued on their prescribed diets of high and low vitamin A content. The results, which are presented in Table I, Experiments 7 to 14, broadly indicate the extent to which vitamin A deficiency may play a part in lowering the animal's resistance to gossypol poisoning.

Eleven rats on the low vitamin A diet were given 25 mg. of gossypol per kilo of body weight. Seven of these were large animals which had been taken off a synthetic diet containing 2 per cent of cod liver oil and placed on a vitamin A-free diet for a period of 5 weeks. At the end of this time growth had practically ceased, but none of the animals had developed xerophthalmia. The four remaining animals were continued on the vitamin A-free diet until the onset of xerophthalmia. Following the injection of gossypol three of this group (Experiment 8) but only two of the former group (Experiment 7) succumbed. Similarly, two of the eight control animals which received the same amount of gossypol succumbed.

Thirteen rats on the low vitamin A diet were then given 38 mg. of gossypol per kilo of body weight. Data for these are presented in Experiments 10, 11, and 12. In the first of this group of experiments, two animals in the advanced stages of xerophthalmia were used. Both succumbed. In the next, six animals with slight xerophthalmia and unable to make gains on the vitamin A-free diet were used. These succumbed almost immediately to the gossypol injection. In the last experiment, five animals without xerophthalmia but which were just beginning to lose weight were used. Only one survived. Similarly, one of the five animals which served as controls for this group survived. Postmortem findings were essentially the same as those described in the vitamin B experiments.

These results are not to be interpreted for the present as meaning that there exists a specific relation between vitamin A and

gossypol, for it is well known that during avitaminosis, rats show an increased susceptibility to certain types of infection and disease quite unrelated to gossypol poisoning. It is perhaps significant, however, that fatalities occurred early and were of frequent occurrence among the vitamin A-depleted animals.

Oral Administration of Gossypol—The following experiments were undertaken in order to determine if the effects of gossypol ingestion are more or less pronounced according to the level at which vitamin A is supplied in the diet. Young rats 30 days old and weighing between 45 and 50 gm. were caged individually in screen bottom cages and fed upon a vitamin A-free diet until they showed positive signs of xerophthalmia. They were then allowed in addition to the basic diet a daily supplement of butter fat. One group destined to become the low vitamin A group was given a daily supplement of 0.1 gm. of butter fat for a period of 4 weeks during which time the animals were able to establish fairly constant gains in weight of 0.8 to 1.0 gm. per day. Another group received daily 0.4 gm. of butter fat over the same period of time and the animals established a gain of 1.4 to 1.6 gm. per day. Gossypol was then added to the basic diets of both groups at the rate of 200 mg. per 150 gm. of food. Two groups of vitamin A-depleted animals were carried along at the same time to serve as controls. Neither of these two received butter fat and only one received gossypol, which was added to the diet in the same proportions as above. The data obtained in these experiments are presented in Table II (Experiments 1 a, 2 a, 3 a, and 4 a).

It is to be noted that death occurred much sooner among the avitaminotic animals in Group 3 a, whose diets contained gossypol, than among any of the others. However, the span of life for animals in a similar stage of vitamin depletion (Group 4 a) and not receiving gossypol was also short. Only one death occurred among the animals receiving 0.4 gm. of butter supplement daily whereas all the animals receiving only 0.1 gm. died in less than 30 days.

Because of difficulty in the interpretation of results of studies in which two dietary factors are involved, in this case gossypol and vitamin A, these experiments were repeated with slight modification. Instead of using animals whose stores of vitamin A had been completely depleted, larger animals were used and their stores

TABLE II

Influence of Vitamin A upon Response of Rats to Gossypol Ingestion

Group or Experiment No.	Rat No.	Initial weight	Final weight	Days on experiment*	Average daily gain or loss	Average daily food intake	Remarks concerning preliminary feeding and condition of animals
Basic diet + gossypol + 0.4 gm. butter fat daily							
1 a	32 ♀	113	75	26	-1.5	4.0	4 wks. on basic diet followed by 4 wks. with butter supplement. Average daily gain, 1.5 gm.
	33♂	110	73	44	-0.8	4.5	
	35♂	115	76	44	-0.8	4.5	
1 b	163♂	110	88	44	-0.5	3.3†	3 wks. on basic diet. Average final 5 day gain, 5.0 gm.
	164♂	85	55	44	-0.7		
	165♂	75	55	15	-1.3		
Basic diet + gossypol + 0.1 gm. butter fat daily							
2 a	28 ♀	100	70	30	-1.0	4.7	4 wks. on basic diet followed by 4 wks. with butter supplement. Average daily gain, 0.9 gm.
	29♂	107	78	22	-1.3	4.7	
	30♂	102	70	13	-2.5	5.0	
Basic diet + gossypol							
3 a	112 ♀	57	45	14	-0.9	3.0	3 wks. on basic diet. Growth had ceased. Slight xerophthalmia
	113 ♀	52	47	5	-1.0	5.5	
	114♂	67	54	8	-1.6	4.7	
3 b	160♂	90	67	10	-2.3	2.7	3 wks. on basic diet. Average final 5 day gain, 5.7 gm.
	161 ♀	98	67	44	-0.7	4.0	
	162♂	85	70	5	-3.0	2.7	
Basic diet							
4 a	115 ♀	63	53	14	-0.7	4.0	3 wks. on basic diet. Growth had ceased. Slight xerophthalmia
	116♂	65	56	14	-0.6	4.0	
	117 ♀	57	47	13	-0.8	4.0	
4 b	168♂	100	92	44	-0.2	6.5†	3 wks. on basic diet. Average final 5 day gain, 5.0 gm.
	169♂	95	115	44	+0.5		
	171 ♀	110	108	44			
Basic diet + gossypol + 0.4 gm. Crisco							
5	190♂	93	60	44	-0.7	2.3†	4 wks. on basic diet. Growth had practically ceased. No xerophthalmia.
	195 ♀	88	56	44	-0.7		
	197♂	95	54	35	-1.2		

* Only those animals which were on experiment for 44 days survived.

† Average values calculated from the food intake of the entire group.

of vitamin A only partially depleted. When placed on experiment these animals were maintaining their weight and showed no sign of xerophthalmia. Gossypol additions were made as before without the preliminary feeding of vitamin A supplement. These experiments constitute the Series b of Table II. As in the previous experiments, two of the animals in Group 1 b, which received gossypol and 0.4 gm. of butter fat daily, survived the experimental period. Only one animal survived in Group 3 b, which received gossypol but no vitamin A supplement, whereas all survived in the control group, Group 4 b, which were in a similar stage of vitamin depletion at the beginning of the experimental period and were continued on the basic diet alone. The ability of the animals in the latter group to maintain almost constant weight during this period of 44 days suggests that all of the animals in Series b had considerable reserve of vitamin A. Furthermore, xerophthalmia did not appear among these animals until after the 30th day of the experimental period. These facts do not necessarily suggest that the toxic action of gossypol may be related to vitamin A avitaminosis, *per se*, but rather suggest that the lower plane of nutrition brought about by withholding vitamin A decreased the animal's resistance to gossypol poisoning. The daily supplement of butter fat constituted approximately one-tenth of the total food consumption of the animals in Groups 1 a and 1 b and was undoubtedly a factor which contributed to their well being. Experiment 5 in which the animals received Crisco in place of butter fat was carried out with this mind. Unfortunately, the animals used in Experiment 5 were not selected from the same stock as those used in Experiments 1 to 4. They grew more slowly during the preliminary feeding period on a vitamin A-free diet. However, when placed on the gossypol diet with a daily supplement of Crisco, they survived as long as those which received the same amount of butter supplement.

In evaluating the results of these experiments, it is of significance to note that all of the animals which received the same amount of gossypol in their diets and were able to survive for more than 44 days lost about the same amount of weight irrespective of their condition as regards vitamin A (see Rats 33, 35, 163, 164, 161, 190, and 195). The early deaths of Rats 165 and 30, which received vitamin A in their diets, and Rats 160 and 162, which were only

partially depleted with respect to vitamin A at the beginning of the experiment, suggest an acute form of gossypol injury in contrast to the delayed or so called chronic injury. The animals in Groups 3 a and 4 a which were in the final stages of avitaminosis also succumbed shortly after the experiment started, although death in these instances was more likely due to vitamin deficiency than gossypol injury.

In the foregoing experiments the amount of gossypol which each animal received was determined by the food intake. Most of the animals were eating close to 4 gm. of food per day, which represents a daily consumption of 5 mg. of gossypol. In order that all of the animals might receive equal amounts of gossypol, it would be necessary either to regulate the food intake or to administer the gossypol separately from the basic diets. This latter method was used in the next group of experiments and the gossypol given by stomach tube in the manner previously described.

Rats weighing between 70 and 80 gm. were removed from a stock diet containing cod liver oil and placed on a vitamin A-free diet for a period of 4 weeks. At the end of this time they were making only slight gains in weight but were not showing signs of xerophthalmia. Each animal was then given 1 cc. of gossypol extract containing 20 mg. of gossypol. An equal amount of gossypol was given to control animals of approximately the same weight as the experimental ones and receiving the same diet supplemented with cod liver oil. After 5 days the animals which survived were given 1 cc. more of the extract. The data are presented in Table III as Experiment 18. Preliminary experiments had shown that normal animals were able to tolerate relatively large doses of gossypol when administered a little at a time by the stomach tube method. Consequently, the rather sudden deaths of animals on the low vitamin A diet in these experiments were surprising. The experiment was repeated and the data presented under Experiment 19. To determine if the cottonseed oil itself might be toxic to the animals on the low vitamin A diet, experiments were conducted in the same way with use of refined cottonseed oil which is free of gossypol. Other animals on the low vitamin A diet were given an equal amount of olive oil but in neither case did the oil produce toxic symptoms.

In Experiment 18 of the eight animals on the low vitamin A

diet, which received 20 mg. of gossypol, four died within the next 3 days. The remaining four which received a second dose of 20 mg. on the 6th day died within the next 2 days. In almost all cases the gossypol produced diarrhea and so affected the appetite that death followed partly no doubt as a result of fasting. For the first 3 or 4 days following the gossypol feeding the experimental animals ate practically no food whereas each of the control animals during the same period consumed between 2 and 3 gm. of food daily. The results of Experiment 19 were of the same order as the preceding ones, and with them offer the most convincing evidence of the increased susceptibility of animals on the low vitamin A diet to gossypol injury. Autopsies of the animals which died showed intestinal inflammation and in a few there was congestion of the lungs.

Experiments with Rats on Low Vitamin D Diets

In the experiments which follow, the high Ca-low P ricketogenic diet, Diet 2965, described by Steenbock and Black (19) was used. This diet is composed of yellow corn 76, wheat gluten 20, CaCO_3 3, and NaCl 1. The experimental animals were kept in semidarkness and only those which grew rapidly during the first 2 weeks on the ricketogenic diet were used. Under the prevailing conditions of the experiments it was found that typical rickets as shown by x-ray pictures was produced in about 3 weeks. Control animals were given Diet 2965 fortified with 3 per cent of cod liver oil and kept in a well lighted room. As in the preceding experiments the animals were given gossypol by intraperitoneal injection, by stomach tube, and as a constituent of the diet after 3 or more weeks on the prescribed diets.

Intraperitoneal Injections—Intraperitoneal injections were made as described and the results recorded in Table I. All of the animals in this experiment appeared to be more susceptible to gossypol when given in this manner than did the animals on the low vitamin A diet in the preceding experiments. Ten of the thirteen rachitic animals which received 25 mg. of gossypol per kilo of body weight died within 8 days and over half of the control animals which received the same amount died in the same length of time. None of the experimental or control animals which received a greater amount of gossypol survived longer than 7 days and only

one survived for more than 5 days after the injections were made. The postmortem findings were essentially the same as those reported in the preceding experiments. One of the animals in Experiment 14, which survived the 8 day experimental period, succumbed on the 15th day. Death resulted from intestinal impaction, a condition not previously observed in this study but reported by Clark (6) as of frequent occurrence among rats whose deaths were delayed following gossypol injections.

The above results indicate that a rachitic condition produced by high Ca-low P diets does not materially decrease the rat's resistance to the toxic effects of gossypol. However, the previous history and dietary regimen of the animals appear to be factors of undetermined influence as judged by the varied response of both avitaminotic and normal animals in the experiments reported in Table I.

Oral Administration of Gossypol—Young rats which had become rachitic on Diet 2965 and control animals which had received the same diet supplemented with 3 per cent of cod liver oil were given 100 mg. of gossypol by stomach tube over a period of 17 days. Although it was first planned to give no more than 20 mg. of gossypol at any one time, it soon became apparent that this was not sufficient to produce immediate fatal results. Consequently, a double amount of gossypol was given on the 17th day. Following this, four of the experimental and three of the control animals died after developing severe diarrhea. The days on which gossypol was given and the weights of the animals are given in Table IV, Experiment 20. In contrast to the results obtained with rats on low vitamin A diets, these rats were able to tolerate relatively large amounts of gossypol and three of the eight rachitic animals lived through the experimental period of 30 days.

The experiment was then repeated with another group of animals treated in a similar way except that a double dose of gossypol was given the 1st day and single doses thereafter until 140 mg. had been given over a period of 30 days. None of the five experimental animals and only two of the five controls survived. It is noteworthy that several of the animals were able to withstand three times as much gossypol as was necessary to bring death to the animals on low vitamin A diets. Furthermore, the amount of food consumed by the animals although negligible immediately follow-

ing the administration of gossypol increased daily so that fasting was less involved.

In the next series of experiments, the results of which are presented in Table V, gossypol was given as a constituent of the diet. Experiments 6 a and 7 a were performed first. Preliminary to the gossypol feeding the animals in these two experiments were fed on Diet 2965 for a period of 4 weeks. Gossypol was then added to the diet of each at the rate of 200 mg. per 150 gm. of food, and cod liver oil to make 3 per cent added to the diet of the animals in Group 7 a. After 52 days on these diets the animals in both groups had lost about the same amount of weight, had consumed practically the same amount of gossypol, and those in Group 7 a had completely recovered from rickets as shown by x-ray pictures. It seemed that these results should warrant the general conclusion that high Ca rickets produces no body changes or functional disturbances which decrease the rat's resistance to gossypol injury. The fact that the animals which received the cod liver oil were able to maintain an apparently normal condition during the greater part of the experiment, except for loss of weight, led to the suggestion that the large amount of calcium carbonate in the diet was an influencing factor. To determine whether or not this was true, the 3 per cent CaCO_3 called for in Diet 2965 was omitted from the diet of rachitic rats and gossypol was added in the same amounts as above. This change in the diet brought fatal results in less than 14 days. The data are presented as Experiments 8 a and 8 b in Table V.

The above experiments were repeated and other experiments designed to serve as controls. One control group of animals after receiving the ricketogenic Diet 2965 for a period of 4 weeks was allowed a modified diet made up the same as Diet 2965 with the omission of the CaCO_3 . This alteration in the diet was in itself sufficient to cause all of the animals to stop growing and one to lose considerable weight and undoubtedly constituted a contributing factor in the early deaths of the animals in Experiments 8 a and 8 b. Two other groups were treated similarly with the exception that the animals were protected against rickets with cod liver oil, and one group received gossypol during the experimental period. The former group made rapid and consistent gains throughout, whereas, the latter showed varied response. Of the animals in the

10	Dead	75	87	Dead	90	92	Dead	Dead	Dead
11									
15			83						
19	20	Dead	76		95	79			
20	20		Dead						
23	20				104	82			
27	20				98	82			
33					93	77			

TABLE V

Influence of Vitamin D upon Response of Rats to Gossypol Ingestion

The basic diet was Steenbock's ricketogenic Diet 2965. The modified basic diet was the same without the calcium carbonate.

Group or Experiment No	Rat No.	Initial weight	Final weight	Days on experiment*	Average daily gain or loss	Average daily food intake	Remarks concerning preliminary feeding period
Basic diet + gossypol							
6 a	80♂	110	77	52	-0.6	6.0	4 wks. on basic diet. Average daily gain, 1.3 gm.
	81♂	115	84	52	-0.6	6.0	
	82♂	107	84	52	-0.4	6.0	
6 b	250♂	84	64	38	-0.5	4.6	4 wks. on basic diet. Average daily gain, 0.7 gm.
	252♂	68	46	38	-0.5	4.2	
	254♂	63	67	40	+0.1	4.2	
Basic diet + cod liver oil + gossypol							
7 a	83♂	100	80	52	-0.4	6.0	4 wks. on basic diet. Average daily gain, 0.8 gm.
	84♂	90	66	52	-0.5	6.0	
	85♂	100	80	52	-0.4	6.0	
7 b	300♀	116	110	40	-0.1	6.8†	4 wks. on basic diet and cod liver oil. Average daily gain, 1.0 gm.
	142♂	90	65	30	-0.8		
	144♀	86	62	31	-0.8		
Modified basic diet + gossypol							
8 a	131♀	87	60	13	-2.0	2.3	3 wks. on basic diet. Average daily gain, 0.9 gm.
	134♂	85	59	7	-3.7	2.0	
	135♀	77	57	7	-2.9	1.0	
8 b	184♀	73	56	7	-2.4	4.0	4 wks. on basic diet. Average daily gain, 0.8 gm.
	187♀	85	70	10	-1.5	3.3	
	251♂	68	47	18	-1.2	3.1	
Modified basic diet							
9	178♂	66	60	40	-0.1	4.7	4 wks. on basic diet. Average daily gain, 0.8 gm.
	179♂	73	65	40	-0.2	4.7	
	186♀	73	60	40	-0.3	4.9	

* Only those animals which were on experiment for 40 days or longer survived, with the exception of Group 12.

† Average values calculated from the food intake of the entire group.

TABLE V—*Concluded*

Group or Experiment No.	Rat No.	Initial weight	Final weight	Days on experiment*	Average daily gain or loss	Average daily food intake	Remarks concerning preliminary feeding period
Modified basic diet + cod liver oil							
10	149♂	109 gm.	192 gm.	40	+2.0	} 9.6†	4 wks. on basic diet and cod liver oil. Average daily gain, 1.2 gm.
	150♀	77	135	40	+1.5		
	151♂	97	167	40	+1.8		
Modified basic diet + cod liver oil + gossypol							
11	301♂	123	130	40	+0.2	7.0	4 wks. on basic diet and cod liver oil. Average daily gain, 1.1 gm.
	147♀	81	58	20	-1.1	2.5	
	148♂	113	67	20	-2.3	2.5	
12	302♀	117	113	25	-0.2	} 5.0†	10 days on basic diet and cod liver oil
	303♀	113	113	25			
	304♂	117	84	25	-1.3		

latter group one made a small gain, three lost weight, and two died within 20 days. These experiments constitute Series b and Experiments 9 to 12 of Table V.

It appears evident from an inspection of Table V that experimental high Ca rickets of itself does not leave rats more sensitive to gossypol. In fact, only two fatalities occurred among the rachitic animals in Groups 6 a and 6 b. Likewise, two animals in Group 7 b, which were protected against rickets with cod liver oil, succumbed. Also, there were two fatalities in Group 11 which received the modified diet supplemented with cod liver oil. The early deaths of all the animals in Group 8, which occurred soon after the CaCO_3 was omitted from their diet, point to the general conclusion that a serious disturbance in calcium metabolism may leave the animals unable to cope successfully with small amounts of gossypol. Whether this condition is directly related to dietary calcium or, as seems more likely, to the lower plane of nutrition produced by upsetting the Ca:P ratio remains to be determined. It is perhaps significant that the phenomenon was observed only among rachitic animals. It is well known that under such condi-

tions, changes in the amount of dietary calcium are usually accompanied by rapid changes in blood calcium and phosphorus.

DISCUSSION

The results which have been presented quite definitely show that the response of rats to gossypol administration is not only dependent upon the quantity of gossypol and the manner in which it is administered, but also upon the nutritional state of the animals. From a comparison of the results in Table I with those in Tables III and IV it is evident that gossypol exerts a more toxic action when given intraperitoneally in oil solution than when given orally. When given intraperitoneally, less than 50 mg. per kilo of body weight proved fatal in most instances, whereas 4 times this amount when given by stomach tube was tolerated by normal animals. Although no experiments were performed which might account for this disparity of results following the administration of gossypol by the two methods, it seems logical to attribute this difference to the more rapid rate of absorption of gossypol from the peritoneal cavity. Schwartze and Alsberg (18) state that gossypol is more potent by this route than by oral or subcutaneous administration.

Avitaminotic animals responded to the intraperitoneal injections of gossypol in much the same way as controls and the small differences that were noted are not considered to be of sufficient significance for detailed discussion. The fact that nearly all of the animals died soon after the injections were made suggests that gossypol when given in this way exerts its toxic influence before any protective mechanism can be effectively established in the body. Practically nothing is known concerning the manner in which gossypol is detoxicated or disposed of in the animal body. Such information which would have an important bearing upon the problem must necessarily await a more complete understanding of the chemical structure of gossypol.⁵

When gossypol was given by stomach tube, the animals on the low vitamin A diet displayed marked susceptibility as shown by the fact that several of them died within the next 48 hours. It is

⁵ An excellent series of papers by E. P. Clark dealing with the chemistry of gossypol is to be found in this *Journal*, **75**, 725 (1927); **76**, 229 (1928); **77**, 81 (1928); **78**, 159 (1928); and *J. Am. Chem. Soc.*, **51**, 1475, 1479 (1929).

unlikely that this result was accidental although no theory is at hand to explain the result adequately. Some of the animals lived to receive a second dose of gossypol but during the intervening period of 5 days they refused food and fasting became a complicating factor in their death. This effect of gossypol upon appetite has been noted before and constitutes a serious difficulty in studies of gossypol tolerance (1). Neither the normal animals nor those made rachitic on high Ca diets deficient in vitamin D showed susceptibility to gossypol injury to this extent although they displayed symptoms of intestinal irritation accompanied by decreased food consumption.

When gossypol was added to the diets of rats either partially or completely depleted in their body stores of vitamin A, or when the animals were supplied with this vitamin in less than optimal amounts, death was of frequent occurrence. Here again, food intake became a complicating factor, the amount of food eaten determining the amount of gossypol received and no doubt otherwise influencing the general condition of the animals. It is only by considering these results as a whole that a general conclusion relating vitamin A deficiency with increased sensitivity to gossypol seems justified. The variations in response of the animals and other factors which cannot be controlled experimentally are recognized as vulnerable points in any argument which might be presented in support of such a conclusion.

Sufficient data are not at hand to warrant a detailed discussion of gossypol injury in relation to calcium and phosphorus metabolism. The fatal results which attended the removal of calcium carbonate from the diet of rachitic rats at the time gossypol was added suggest that the physiological changes which take place during rickets and following such alterations in the diet may lead to increased susceptibility to gossypol injury. It is planned to investigate this point further in its possible relation to the use of cottonseed meal in rations unbalanced in respect to mineral content.

SUMMARY

The response of rats to gossypol administration during vitamin A, vitamin B complex, and vitamin D avitaminosis has been observed and compared to that of normal rats receiving adequate

diets and equivalent amounts of gossypol. The gossypol was administered by intraperitoneal injection in amounts which represented 25 and 38 mg. per kilo of body weight; by stomach tube in 20 mg. doses; and by incorporating it in experimental diets to make about 0.13 per cent.

Only small differences were noted in the response of the avitaminotic and normal animals to the intraperitoneal injections which barely indicated that those on low vitamin A diets were more sensitive to gossypol when given by this route than were the normal controls. Edema and intestinal inflammation usually followed the injections.

When gossypol was administered by stomach tube it produced diarrhea accompanied by loss of appetite and probably other disorders which contributed to the early deaths of many of the rats on low vitamin A diets. Normal animals and rachitic ones were less affected and were able to withstand much larger quantities of gossypol before they succumbed to this treatment.

When gossypol was given as a constituent of the diet practically all of the animals declined and a few which survived for more than 40 days lost weight at about the same rate irrespective of their condition as regards vitamin depletion. As a rule the rats which received suboptimal amounts of vitamin A or which were partially depleted in their body stores of this vitamin previous to and during the feeding of gossypol diets, displayed greater sensitivity to gossypol than did control animals.

In experiments with rachitic rats on gossypol diets, it was found that the removal of excess calcium from the diet was attended by fatal results.

These findings are believed to be of importance in their relation to the fatal results so often encountered in the common use of cottonseed products in rations undoubtedly deficient in certain vitamins and perhaps unbalanced in mineral content.

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NOTE CONCERNING THE α - AND β -GLYCERO-PHOSPHATES

BY P. KARRER AND H. SALOMON

(From the Chemical Institute, University of Zurich, Zurich, Switzerland)

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Kay and Lee have published in this *Journal*¹ an investigation regarding the hydrolysis of α - and β -glycerophosphate. For the determination of the α and β form they used among other methods also the one proposed by us,² which is based upon the fact that only the β -glycerophosphate renders with barium nitrate a double salt, relatively insoluble in water. For our investigations we occasionally used a 10 per cent solution of α - and β -barium glycerophosphate and Kay and Lee state that they were unable to prepare solutions of such a concentration, as the solubility in water of about 25° amounts for the barium α -glycerophosphate only to 1.7 per cent and for β -salt only to 5.1 per cent. These authors overlooked the fact that the solutions prepared by us were supersaturated solutions and that we described their method of preparation in detail.³ We stated repeatedly that the real solubility of each of the two barium glycerophosphates is considerably smaller and have given exact figures for their respective rates of solubility.⁴ Mixtures of α - and β -barium glycerophosphates show a greatly increased rate of solubility.

To precipitate β -barium glycerophosphate from a solution by means of barium nitrate, solutions of a lesser concentration than 10 per cent may also be used, as the solubility of the barium nitrate double salt in water of about 20° is only 0.8 to 1 per cent.

If supersaturated solutions of the barium glycerophosphates are being used for the precipitation with barium nitrate, the resulting precipitate must of course be tested for nitrogen, in the manner described by us.³

¹ Kay, H. D., and Lee, E. R., *J. Biol. Chem.*, **91**, 135 (1931).

² Karrer, P., and Salomon, H., *Helv. Chim. Acta*, **9**, 3 (1925).

³ Karrer, P., and Benz, P., *Helv. Chim. Acta*, **10**, 89 (1926).

⁴ Karrer, P., and Salomon, H., *Helv. Chim. Acta*, **9**, 3, 9, 10, 599 (1925).

NOTE CONCERNING THE α - AND β -GLYCERO-PHOSPHATES

A REPLY TO KARRER AND SALOMON

By H. D. KAY

(From the Department of Biochemistry, University of Toronto,
Toronto, Canada)

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The papers of Karrer and his coworkers mentioned in Karrer and Salomon's note¹ were read by us with great interest some years ago.

Karrer and Salomon agree with our implication² that a 10 per cent aqueous solution of the barium salt of either α - or β -glycerophosphoric acid is greatly supersaturated. We ourselves feel that it is dangerous on general grounds to use supersaturated solutions for precipitation reactions. Thus without an analysis for nitrogen it would be unwise to conclude that a precipitate obtained by adding an equal volume of a solution of barium nitrate to a 10 per cent solution of an unknown barium glycerophosphate indicates the presence of the β -isomer, since a precipitate will be obtained in any case on keeping a 5 per cent (*i.e.* supersaturated) solution of barium α -glycerophosphate for the length of time (12 hours) recommended by Karrer and coworkers for the precipitation of the double compound between barium nitrate and barium β -glycerophosphate. In our experience not only is it not easy to obtain such grossly supersaturated solutions of either pure barium α - or pure barium β -glycerophosphate, but both salts will form precipitates in a few hours in their supersaturated solutions, with or without the addition of barium nitrate.

This statement of ours, like that in the paper to which Karrer and Salomon refer,² is not of course intended to detract in any way from the real value of Karrer's method, but to indicate that the method must be used with considerable discretion.

¹ Karrer, P., and Salomon, H., *J. Biol. Chem.*, **93**, 407 (1931).

² Kay, H. D., and Lee, E. R., *J. Biol. Chem.*, **91**, 135 (1931).

THE EFFECTS OF HIGH AND PROLONGED MAGNESIUM LACTATE INTAKE UPON THE METABOLISM OF MAGNESIUM AND CALCIUM IN MAN*

BY HARRY E. CARSWELL AND JAMES E. WINTER

(From the Department of Physiological Chemistry and the Department of Physiology and Pharmacology, University of Louisville, Louisville)

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In connection with the growing therapeutic use of magnesium, especially over long periods of time, it is of importance to determine the effects of such use on the calcium and magnesium metabolism in man.

Hitherto, agreement has not been reached as to the effects of magnesium on the calcium metabolism, one widely prevailing impression being that magnesium has a tendency to displace calcium from the body. This is based largely on the results of subcutaneous injections of magnesium salts in animals which Mendel and Benedict (1) found to increase the calcium loss through the urine.

Later workers have found with the ingestion of magnesium salts conflicting results, and the present condition of our knowledge is well reviewed by Elmslie and Steenbock (2). They conclude from their results and those of others that in the presence of adequate phosphorus in the diet there is no calcium loss from the body as a result of short courses of magnesium administration in man and longer courses in growing rats.

Recently Barbour and Winter (3) administered magnesium in the form of gluconate and lactate to two pairs of dogs over periods of 19 to 63 days, and were able to demonstrate a definite calcium retention in the pair receiving the higher phosphorus intake, although calcium loss was observed in the other pair of dogs.

* In cooperation with the Pharmaceutical Division of the Calco Chemical Company, Inc., Bound Brook, New Jersey.

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In the present work, magnesium lactate was administered on account of its relatively easy absorbability. As much magnesium lactate was given as could be tolerated without undue laxative action. Care was taken to provide adequate phosphorus in the diet.

The chief objective was the question of calcium and magnesium balance, but data were also sought as to the paths of excretion of calcium, magnesium, and phosphorus under the conditions of an excess magnesium intake.

EXPERIMENTAL

Two healthy, slightly obese men were the subjects upon whom the metabolism tests were conducted. J. E. W., weighing 88 kilos, and H. E. C., weighing 87.7 kilos, were fed for 37 and 28 days respectively on a uniform diet. A mixed diet was selected which was expected to satisfy the need for protein, fat, carbohydrate, vitamins, and mineral constituents. Special attention was given to the character of the mineral constituents, and foodstuffs were selected which would furnish adequate calcium, magnesium, iron, and phosphorus.

The diet consisted of bran cereal, milk, oranges, bread, butter, lean beef, ham, potatoes, cabbage, beets, carrots, cheese, ice cream, water, sodium chloride, and sugar. The foods were accurately weighed at each meal and the whole allowance was consumed. The number of 100 calorie portions of food in the daily diet follows.

Cereal....1	Butter. . . 3	Cabbage.. .0.5	Ice cream...2
Milk... . . 1.5	Ham 1	Beets... . . 0.56	Sugar..... .3
Orange.....1	Beef... . . 2	Carrots....0.56	NaCl..... .
Bread.....4	Potato... . 1 2	Cheese... . 1.36	Water

This gave as the estimated caloric value of the diet 2268. Typical diet samples were analyzed and the intake figures for MgO, CaO, and P₂O₅ are included in Tables I to III. The beef was served as steak and as a roast with the excess fat removed. The potatoes were baked and the cabbage was served raw. Carrots and beets were boiled, diced, and served with salt and butter. The ham was boiled and the excess fat removed. The other constituents of the diet were procured under the same individual brand in order to maintain the diet as uniform as possible through-

out the experimental period. This daily diet was estimated to furnish 60 gm. of protein, 94 gm. of fat, and 300 gm. of carbohydrate, and a caloric value of 2268. At different intervals during the experimental period, a complete daily allowance of food was accurately weighed, ashed in a muffle furnace, and the content of calcium, magnesium, and phosphorus determined. The methods used for these determinations were the "Methods of analysis of the Association of Official Agricultural Chemists." The amounts as determined were calcium 1.74 gm. as CaO, magnesium 0.58 gm. as MgO, and phosphorus 3.39 gm. as P_2O_5 .

TABLE I

Average Daily Metabolism of Ca and Mg in Subject H. E. C. before, during, and after Mg Lactate Administration

Period	Days	CaO output per day			CaO intake per day	CaO balance	MgO output per day			MgO intake per day	MgO balance
		Urine	Feces	Total			Urine	Feces	Total		
		gm.	gm.	gm.	gm.		gm.	gm.	gm.	gm.	
1st control	3	0.20	1.50	1.70	1.74	+0.04	0.26	0.39	0.65	0.58	-0.07
1st Mg.	6	0.17	0.85	1.02	1.74	+0.72	0.35	1.36	1.71	1.82	+0.11
2nd "	6	0.19	0.90	1.09	1.74	+0.65	0.38	0.81	1.19	1.82	+0.63
3rd "	4	0.19	1.01	1.20	1.74	+0.54	0.34	1.13	1.47	1.82	+0.35
4th "	4	0.20	0.81	1.01	1.74	+0.73	0.47	0.92	1.39	1.82	+0.43
Final control.	3	0.23	1.30	1.53	1.74	+0.21	0.24	0.31	0.55	0.58	+0.03

Total retention of CaO during 20 days, 13.30 gm.; total retention of MgO during 20 days, 7.56 gm.; total CaO absorbed (net) during 20 days, 17.20 gm.; total MgO absorbed (net) during 20 days, 15.18 gm.

Each subject was fed 2 days on the uniform diet before any collections of excreta were made. The first experimental period was a control period, during which time the uniform diet alone was consumed. A high magnesium diet then was consumed by J. E. W. for 24 days, while H. E. C. consumed the same diet for 20 days. The high magnesium diet was obtained by adding to the uniform diet, daily, 8 gm. of magnesium lactate, which were equivalent to 1.24 gm. of MgO. Following these long periods of high magnesium intake, a final control period was conducted, during which time each subject eliminated the 8 gm. of magnesium lactate from the daily diet and consumed the uniform diet alone as in the first control period.

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TABLE II

Average Daily Metabolism of Ca and Mg in Subject J. E. W. before, during, and after Mg Lactate Administration

Period	Days	CaO output per day			CaO intake per day	CaO balance	MgO output per day			MgO intake per day	MgO balance
		Urine	Feces	Total			Urine	Feces	Total		
		gm.	gm.	gm.	gm.		gm.	gm.	gm.	gm.	
1st control....	3	0.36	0.97	1.33	1.74	+0.41	0.29	0.25	0.54	0.58	+0.04
2nd "	3	0.33	0.73	1.06	1.74	+0.68	0.25	0.26	0.51	0.58	+0.07
1st Mg.....	3	0.32	0.93	1.25	1.74	+0.49	0.46	0.35	0.81	1.82	+1.01
2nd "	2	0.35	0.54	0.89	1.74	+0.85	0.36	0.42	0.78	1.82	+1.04
3rd "	6	0.23	1.45	1.68	1.74	+0.06	0.43	0.72	1.15	1.82	+0.67
4th "	6	0.27	1.31	1.58	1.74	+0.16	0.57	0.88	1.45	1.82	+0.37
5th "	7	0.28	1.35	1.63	1.74	+0.11	0.32	0.80	1.12	1.82	+0.70
Final control.....	7	0.33	1.56	1.89	1.74	-0.15	0.33	0.61	0.94	0.58	-0.36

Total retention of CaO during 24 days, 5.26 gm.; total retention of MgO during 24 days, 16.25 gm.; total CaO absorbed (net) during 24 days, 11.88 gm.; total MgO absorbed (net) during 24 days, 26.59 gm.

TABLE III

Average Daily Metabolism of Phosphorus before, during, and after Mg Lactate Administration

Subject	Period	P ₂ O ₅ output			P ₂ O ₅ intake	Balance
		Feces	Urine	Total		
		gm.	gm.	gm.	gm.	
J. E. W.	1st control	2.63	2.16	4.79	3.39	-1.40
	2nd "	2.32	2.31	4.63	3.39	-1.24
	1st Mg.....	0.66	1.14	1.80	3.39	+1.59
	2nd "	1.16	1.50	2.66	3.39	+0.72
	3rd " . . .	1.28	1.72	3.00	3.39	+0.39
	4th " . . .	1.28	1.51	2.79	3.39	+1.60
	5th "	1.23	1.17	2.40	3.39	+1.99
	Final control	1.46	2.37	3.83	3.39	-0.50
H. F. C.	1st control	1.12	2.22	3.34	3.39	+0.05
	1st Mg . . .	0.87	1.93	2.80	3.39	+0.59
	2nd "	0.69	1.94	2.63	3.39	+0.76
	3rd " . . .	0.28	1.89	2.15	3.39	+1.24
	4th "	0.59	1.88	2.47	3.39	+0.92
	Final control	0.87	2.06	2.93	3.39	+0.40

Total retention of P₂O₅ in subject J. E. W. for 24 days, 18.60 gm.

Total retention of P₂O₅ in subject H. E. C. for 20 days, 16.64 gm.

The experimental periods were marked off in the feces by the taking of carmine just before the first meal of the period. The feces were collected in waxed containers and dried. The dried feces were combined in periods, ground, thoroughly mixed, and ashed in a muffle furnace. Urine collections were made daily and combined in periods. Before analysis the urine specimens were made acid and filtered.

Calcium and magnesium in the urine and feces were determined gravimetrically by McCrudden's method (4). Phosphorus in the urine and feces was determined by the uranium acetate titration method.

Both subjects remained in good health during the entire course of the experiment. The high magnesium content of the diet due to the ingestion of magnesium lactate had a pronounced but not disturbing laxative action upon both subjects. The stools during the periods of 24 and 20 days respectively were very fluid in consistency.

DISCUSSION

Control or Normal Period—An examination of the data in Tables I and II shows that both subjects were in positive calcium balance during the first control or normal period, indicating that the calcium requirement was being met. Subject J. E. W., however, was retaining more calcium than subject H. E. C. and his urinary calcium was also unusually high during his control periods.

Both subjects were practically in magnesium balance during this period, indicating that the magnesium requirement had been met. The urinary calcium was higher than the urinary magnesium in one subject, but lower in the other subject. This is in agreement with the observations of Bogert and McKittrick (5), and Nelson and Burns (6), who concluded that either calcium or magnesium may be in excess in the urine, and which ever predominates does so consistently in each individual.

The fecal magnesium was less than the fecal calcium in both subjects for the first period. The ratio varied from 1:3 to 1:3.8 and is much higher than that observed by Bogert and McKittrick (5), but probably it is due to the fact that the diets which they used in their experiments were much lower in calcium content than the diet used in these experiments. Our results confirm

observations made by Givens and Mendel (7), that calcium is always in excess of magnesium in the feces. The total magnesium excretion was also found less than the total calcium excretion in both subjects during the normal period. The ratio varies from 1:2.2 to 1:2.6.

The ratio of the urinary calcium to fecal calcium during the first control periods varied from 1:2.4 to 1:7.5, while the ratio of the urinary magnesium to fecal magnesium during the same periods varied from 1:0.94 to 1:1.5. The latter is somewhat lower than the 1:2 ratio given by Underhill, Honeij, and Bogert (8), but the urinary calcium: fecal calcium ratio agrees with their observations that there is a wide variation in the relation of calcium in the urine and feces of normal individuals.

The above observations, aside from the fact that subject J. E. W. was retaining calcium in relatively large amounts during the control period, place the calcium and magnesium metabolism of our control periods in agreement with the observations of other investigators.

Effects of Magnesium Lactate—In the case of subject H. E. C. there was a very definite decrease in fecal calcium and a very slight decrease in urinary calcium, immediately following the ingestion of a relatively large amount of magnesium lactate. After 6 days of the high magnesium diet, both the urinary and fecal calcium increased slightly and remained at about the same respective levels until the magnesium lactate was dropped from the diet, when the fecal calcium increased to a level near that of the first control period. There was very little change in the level of the urinary calcium. The net CaO absorbed during 20 days of a high magnesium diet was 17.2 gm., with a total retention during that period of 13.3 gm. of CaO.

In the case of subject J. E. W., for 5 days after the first ingestion of the high magnesium diet, the urinary calcium remained about the same as in the control period, the increased absorption of CaO with subsequent high retention continuing. With the beginning of the second lactate period, the urinary calcium decreased slightly and maintained a uniform level throughout the remainder of the period of high magnesium intake. The fecal calcium rose rapidly during the 2nd week of high magnesium intake until the total output was just slightly less than the intake. This level also was

then uniformly maintained. The total net absorption of CaO during the 24 days of the high magnesium diet was 11.8 gm. with a total retention of 5.26 gm. for the same period.

The above data indicate that the ingestion of a very high magnesium diet with an adequate phosphorus content, does not materially affect the urinary calcium over a long period of time. Furthermore calcium storage need not be decreased and may be decidedly augmented by such a magnesium intake.

The fecal calcium in one case was materially decreased with a definite retention of calcium, while in the other case, the fecal calcium was increased over that of the control period, yet the subject was always in positive calcium balance for a period of 24 days.

Magnesium Metabolism—On examining Table I it is noted that the ingestion of an increased amount of magnesium increased both the urinary and fecal magnesium, which was to be expected from such an abnormally high content of magnesium in the diet. Table II shows the same general effect; subject J. E. W. had, however, a net absorption of 26.6 gm. of MgO during 24 days with a retention of 16.25 gm. of MgO , while H. E. C. during 20 days, had a total net absorption of MgO of 15.18 gm. with a total retention of 7.56 gm. of MgO . Thus the two subjects absorbed respectively 61 and 42 per cent of their magnesium intake during the high magnesium administration.

On the basis of the average daily retention of calcium, magnesium, and phosphorus during the high magnesium experimental period, J. E. W. retained, daily, 0.68 gm. of MgO , 0.22 gm. of CaO , and 0.78 gm. of P_2O_5 (Tables II and III) and H. E. C. retained, daily, 0.39 gm. of MgO , 0.66 gm. of CaO , and 0.83 gm. of P_2O_5 (Tables I and III). Thus, while both subjects retained daily about the same amount of phosphorus, one was retaining magnesium at about the same ratio as the other was retaining calcium.

SUMMARY

1. 8 gm. of magnesium lactate per day were added over periods of 20 and 24 days, respectively, to the uniform, phosphorus-adequate diet of two subjects.

2. Definite calcium retention resulted in one case and a slight calcium retention in the other.

3. Conversely, the magnesium retention in the former case was slight; in the latter it was marked.

4. The urinary calcium was decreased slightly in both cases. The fecal calcium was materially decreased in the case which showed marked calcium retention and increased in the other. The urinary and fecal magnesium were increased in both cases.

CONCLUSIONS

With adequate phosphorus intake magnesium appears to favor calcium storage instead of causing calcium loss. About one-half of the magnesium from a large daily intake of magnesium lactate was readily absorbed in two men.

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CLINICAL CALORIMETRY

XLVII. PROLONGED MEAT DIETS WITH A STUDY OF THE RESPIRATORY METABOLISM

BY WALTER S. McCLELLAN, HENRY J. SPENCER, AND EMIL A.
FALK

WITH THE TECHNICAL ASSISTANCE OF G. F. SODERSTROM

(From the Russell Sage Institute of Pathology in Affiliation with the Second
Medical (Cornell) Division, Bellevue Hospital, New York)

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INTRODUCTION

The effects on two men of an exclusive meat diet lasting 1 year have been discussed in two preceding papers (1, 2). In the course of these studies thirty-five observations of their respiratory metabolism, both in the basal condition and after meals, were made in the Sage calorimeter. These observations form the basis for the present communication.

Experimental Methods and Alcohol Checks

The methods used in the ward and the calorimeter of the Russell Sage Institute of Pathology have been described in earlier

papers (3, 4). The accuracy of the calorimeter data was controlled by frequent alcohol checks, a summary of which (Table I) shows the average results of 3 consecutive hours for each check. The alcohol solution used contained 91.28 per cent of alcohol by weight. The amount burned per hour varied from 9.1 to 11.0 cc. Complete combustion of 10 cc. of this solution should give the following figures: O₂ 15.43 gm., CO₂ 14.14 gm., H₂O 9.39 gm., and heat 52.39 calories. The percentage errors given in Table I

TABLE I
Alcohol Checks

Date	Error, per cent			Average R. Q.*	Date	Error, per cent			Average R. Q.*
	Heat	O ₂	CO ₂			Heat	O ₂	CO ₂	
<i>1927</i>					<i>1928</i>				
Dec. 27	-1.2	+0.1	-0.9	0.661	Oct. 30	-1.4	-3.3	-2.9	0.669
<i>1928</i>					Nov. 13	-1.1	+0.1	-1.0	0.661
Jan. 5	+0.0	+0.3	-1.3	0.655	" 15	-1.0	-1.4	-1.3	0.668
" 18	-1.6	-0.9	-1.2	0.665	" 23	-0.4	-0.4	-0.2	0.667
" 26	-0.6	-0.8	-1.4	0.662	Dec. 13	+0.8	-0.6	-0.9	0.665
Feb. 16	-0.5	-0.9	-0.6	0.668	<i>1929</i>				
Mar. 2	-0.4	-2.2	-1.0	0.675	Jan. 15	+0.1	-1.0	-1.4	0.664
" 13	-1.5	-0.4	-0.6	0.665	Feb. 7	†	+0.7	+0.2	0.664
Apr. 3	-2.2	-0.5	-0.8	0.665	" 28	†	-0.9	-0.8	0.668
May 8	-0.9	+1.1	-0.8	0.655	Mar. 7	-1.4	-0.9	-1.2	0.665
" 9	-1.7	-0.2	-0.9	0.662	" 29	+1.2	-1.4	-0.5	0.672
					Apr. 16	+2.2	-0.1	-0.5	0.665
					May 3	-1.3	-0.5	-0.3	0.668
Average, 1927-28.	-1.1	-0.4	-1.0	0.663	Average, 1928-29.	-0.2	-0.9	-0.9	0.666

* The theoretical R. Q. is 0.667.

† The direct heat was not recorded.

are the variations of the measured quantities from the theoretical amounts as calculated from the volume of alcohol burned. The hourly respiratory quotients of the individual checks showed an average variation of 0.02 with the lowest variation 0.00 and the greatest 0.05,

Experimental Data

The studies in the calorimeter with V. S. as the subject were thirteen in number; seven when he was in basal condition and six

after food. K. A. underwent eighteen observations of which ten were basal determinations and eight following food. Two tests in the basal state and two after meals were carried out with the subject, E.F.D.B. A summary of the data is presented in Table II. Observations of a similar nature have been grouped together for purposes of comparison.

Basal Metabolism

No significant variation in the basal metabolic rates of the men was found. In the basal observations, V. S. ranged between 80

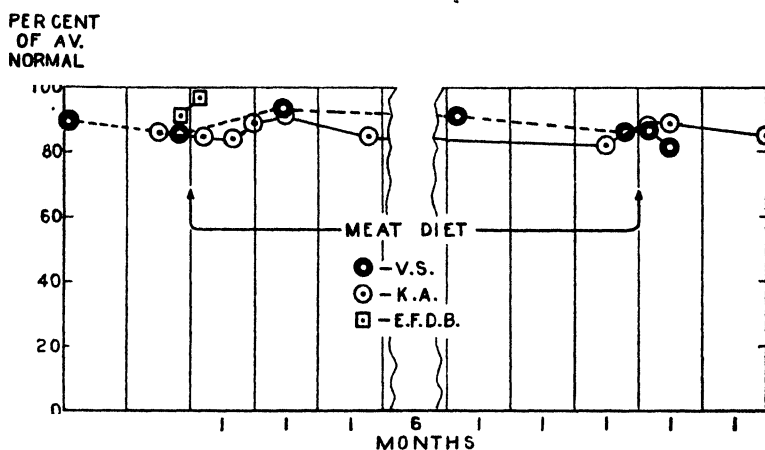


CHART I. The basal metabolic rates of the men who took the exclusive meat diets as calculated from the Aub-Du Bois standards. Each symbol represents the average rate for 3 consecutive hours, 16 to 20 hours after food.

and 91 per cent of normal according to the Sage standards, K. A. varied from 82 to 90 per cent, and E.F.D.B. showed 91 and 97 per cent in two tests. After meat was taken for 6 weeks, the basal metabolism of V. S. was 7 per cent above that found in the period of mixed diet and similarly the rate of K. A. rose 5 per cent. At the end of 10 days on the diet E.F.D.B. showed a rate 6 per cent higher than at the beginning. The basal metabolic rates of V. S. and K. A. at the end of the year were at or below those found at the beginning. These changes are shown graphically in Chart I.

TABLE II
Summary of Data on Respiratory Metabolism (Averages per Hour)

Experiment No., subject, and date	Time	Oxy- gen	R. Q	Uri- nary N ₂	Non- protein R. Q.	Indirect calorimetry		Direct calori- metry	Remarks
						Protein	Total		
Lean meat									
	hrs.	gm.		gm		calories	calories	calories	
1. K. A., Jan. 10, 1928	2	17.29	0.782	0.455	0.776	12.05	56.79	52.35	Basal on mixed diet
2. " " 20, 1928	2	19.82	0.822	0.655	0.828	17.35	65.70	66.13	300 gm. meat. Start 2 hrs. after eating
3. " " 31-Apr. 13, 1928	2	20.78	0.800	0.693	0.798	18.38	68.50	70.55	Five basals during first 2 mos. of meat diet
	15	17.56	0.760	0.533	0.745	14.13	57.40	60.71	
4. K. A., Mar. 23, 1928	2	20.87	0.734	0.928	0.691	24.59	67.32	59.95	End of 2 mos. on meat diet. 300 gm. meat. Start 1 hr. after eating
5. " Jan. 10, 1929	2	21.56	0.766	1.090	0.737	28.90	69.73	73.10	Basal. End 1 yr. on meat diet
6. " " 24, 1929	3	16.55	0.781	0.615	0.771	16.31	54.17	61.53	467 gm. meat. Start 2 hrs. after eating
	2	24.04	0.755	0.954	0.730*	25.28	78.02	68.94	Basal on mixed diet
	2	24.41	0.743	2.124	0.571	56.29	77.50	76.63	500 gm. meat. Start 1 hr. after eating
7. V. S., Feb. 21, 1928	3	18.78	0.803	0.511	0.802	13.55	62.17	60.96	Basal. 1 mo. on meat diet
8. " " 28, 1928	4	22.16	0.806	0.716	0.807	18.98	73.20	69.96	500 gm. meat. Start 2 hrs. after eating
9. " Apr. 12, 1928	3	20.13	0.761	0.651	0.744	17.26	65.72	66.38	Basal. 1 yr. on meat diet
10. " Mar. 20, 1928	4	23.17	0.747	1.126	0.706	29.86	74.92	72.68	400 gm. meat. Start 2 hrs. after eating
11. " " 15, 1929	3	18.75	0.787	0.721	0.777	19.11	61.39	57.73	Basal. 1 yr. on meat diet
12. " " 8, 1929	4	23.53	0.762	0.951	0.740	25.21	76.55	72.12	400 gm. meat. Start 2 hrs. after eating

13. K. A., Jan. 31, 1929	3	17.39	0.733	0.341	0.719	9.04	56.81	56.23	Basal while on high fat diet
14. " " 29, 1929	4	19.71	0.734	0.435	0.718	11.53	64.31	63.88	264 gm. fat. Start 2 hrs. after eating
15. V. S., Mar. 12, 1929	3	18.66	0.769	0.306	0.764	8.12	61.61	68.81	Basal on high fat diet with 35 gm. carbohydrate
16. " " 15, 1929	4	21.40	0.773	0.447	0.766	11.85	70.54	66.18	166 gm. fat. Start 2 hrs. after eating

Night observations									
17. K. A., Apr. 4-5, 1928†	4	19.54	0.733	0.699	0.698	18.54	63.43	64.14	Start 6½ hrs. after supper
18. V. S., " 16-17, 1928†	4	21.21	0.760	0.865	0.736	22.92	68.90	68.88	Start 6 hrs. after supper

Lean meat + fat									
19. K. A., Mar. 6, 1928†	3	19.48	0.761	0.671	0.743	17.79	63.53	62.90	P.c. § breakfast, P 30; F 87. 1 hr.
20. " " 7, 1928†	3	21.47	0.727	0.814	0.691	21.57	69.43	68.12	Breakfast, P 40, F 89; p.c. lunch, P 40, F 89. 1 hr.
21. " Apr. 26, 1928†	3	21.43	0.739	0.909	0.701	24.09	69.40	72.95	Breakfast, P 40, F 54; p.c. lunch, P 40, F 54. 2 hrs.
22. V. S., Mar. 15, 1928†	3	22.05	0.740	0.875	0.707	23.19	71.40	68.70	Breakfast, P 30, F 43; p.c. lunch, P 40, F 54. 1 hr.
23. E. F. D. B., Apr. 6, 1928	3	20.61	0.820	0.529	0.824	14.02	68.59	67.55	Basal mixed diet
24. " " 10, 1928	1	25.06	0.752	0.798	0.733	21.16	81.63	59.51	Breakfast, P 40, F 75; p.c. lunch, P 40, F 74. 1 hr.
25. " " 19, 1928	2	25.82	0.791	0.807	0.786	21.39	85.01	87.36	Basal after 10 days meat diet
26. " " 18, 1928	3	21.71	0.772	0.690	0.759	18.30	71.10	67.76	Breakfast, P 40, F 59; p.c. lunch, P 40, F 59. 1 hr.
	1	24.51	0.794	1.288	0.786	34.15	79.79	83.11	
	2	26.85	0.745	1.338	0.704	35.46	86.66	85.10	

* The non-protein N. of the first 2 hours would be 0.610 if the nitrogen for the second 2 hours is used.

† The basal data for comparison with these observations are given in Experiment 3.

‡ The basal data for comparison with these observations are given in Experiment 9.

§ P.c. indicates after meals, P, protein, and F, fat.

The metabolism of both V. S. and K. A. was at a relatively low level but it was by no means subnormal for such well trained subjects. The increase in the metabolism during the early weeks of the exclusive meat ingestion might have been caused by the increased protein metabolism or by the ketosis which was present. The absence of any elevation at the end of the year showed that the diet did not result in any sustained stimulation of the basal metabolism.

The basal heat output of V. S. was usually between 61 and 65, and of K. A. between 54 and 58 calories per hour. The maintenance requirement or the daily amount of food necessary to keep the men in nutritional equilibrium proved to be approximately 75 per cent above their basal heat production for the 24 hours. When V. S. took food with an energy value of 2700 calories per day, and K. A. 2500 calories, they remained at constant weight for long periods but when they took less they gradually lost weight. The activity of the men was quite constant as they slept and ate in the ward and aside from their movements about the ward they walked from 3 to 5 miles daily.

Specific Dynamic Action of Food

Lean Meat—The metabolism of each subject was studied on three occasions after taking lean meat; first, during the periods when they took mixed diets, second, after 2 months of eating meat alone, and, third, at the end of the year. The amount of meat taken, the hours observed, the relation of the heat production to their basal output, the caloric value of each foodstuff metabolized, and the amount of nitrogen excreted in the urine per hour are graphically presented in Chart II. The observations were started either 1 or 2 hours after the middle of the time required to eat the meat and were all continued for 4 hours. For purposes of comparison only the 3rd, 4th, and 5th hours after the meal have been taken for calculating the extra heat production. Analyses of duplicate samples of the meat eaten averaged 21.3 per cent for protein and 3.0 per cent for fat.

The increase in the metabolism of K. A. after eating 300 gm. of meat, containing 63.9 gm. of protein and 9.0 gm. of fat, averaged 17.3 per cent of his basal heat production in the first observation and 19.0 per cent in the second. Following a larger meal of 467

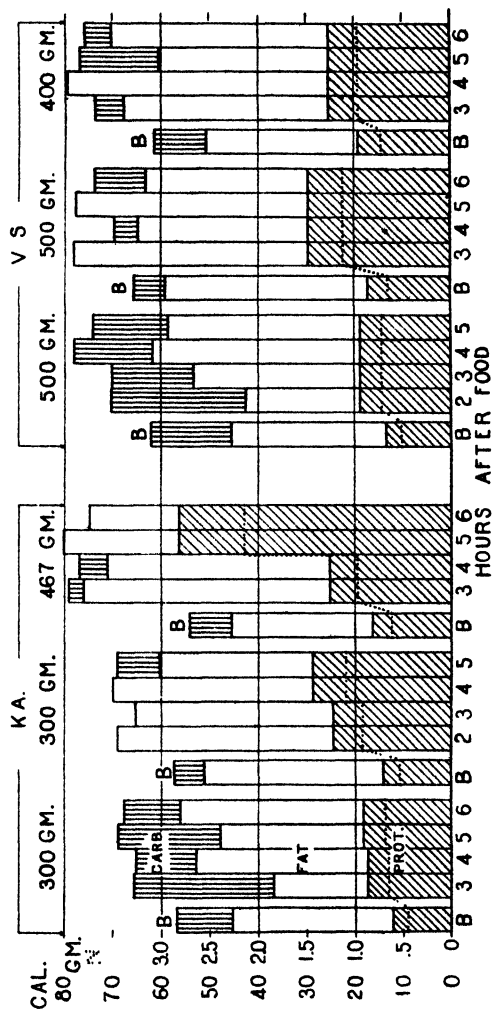


CHART II. The hourly metabolism following lean meat meals. The first test on each subject was made while he was receiving a mixed diet before the special diet was started. The second test was made after 2 months on the meat diet and the third at the end of the year. The amount of meat taken is shown at the top. The dotted lines represent the gm. of urinary nitrogen per hour.

gm. of meat in the third test the rise was 45.3 per cent. It was unfortunate that we did not determine the effects of another meal containing 300 gm. of meat at this time, for, although there were indications that the specific dynamic action was greater, the evidence is not conclusive.

An increase of 19.2 per cent above the basal level occurred in the first observation with V. S. and 14.6 per cent in the second test, in each of which he took 500 gm. of lean meat. After his third meal of 400 gm. of meat the increase was 25.0 per cent. In this subject even the smaller meal at the end of the year caused a greater specific dynamic effect.

The excretion of nitrogen in the urine varied from 0.95 to 1.13 gm. per hour in three of the four observations while they were on the meat diet. In the final test K. A. excreted 2.12 gm. per hour during the last 2 hours of the experiment. At this high rate his protein metabolism could account for 56.3 out of a total of 77.5 calories per hour or 72.6 per cent of his whole metabolism. Only 21.2 calories were left to be divided between fat and carbohydrate and the non-protein respiratory quotient reached the low level of 0.57.

Two explanations for these findings were considered. First, that part of the nitrogen in the urine was actually produced from protein metabolized in preceding periods and due to the lag of its excretion, did not represent the protein metabolism of the same period. Secondly, in a man whose stored glycogen has been depleted, a portion of the glucose obtained from the protein might have been stored rather than burned.

In support of the second explanation the following calculation is presented. Lusk (5), in analyzing the metabolism of protein in patients with severe diabetes who had D:N ratios in the urine of 3.65, showed that under these abnormal conditions the theoretical R.Q. for protein is 0.632 instead of 0.801, which occurs when the protein is completely oxidized. McCann (6), who gave a large meal of meat to a man who had fasted for 8 days, applied this method of calculation to his results and concluded that the glucose which was produced from protein was stored instead of being burned or excreted in the urine. Using the R.Q. for protein of 0.632 in our observations, we found a quotient for the metabolism of fat and carbohydrate of 0.81 for the last 2 hours. This indi-

cated that approximately 5.5 gm. of carbohydrate were oxidized per hour. Theoretically 7.75 gm. of glucose were obtained per hour from the protein metabolism so the remaining 2.25 gm. of glucose must have been stored as no glucose was excreted in the urine. If we assumed that all the glucose oxidized came from protein, we found that 63 per cent of the total energy in this observation was actually derived from protein and the remainder from fat. This level of protein metabolism has only been reached in one other observation on man as far as we know. Aub and Du Bois (7) gave 662 gm. of meat to a dwarf weighing 41 kilos and

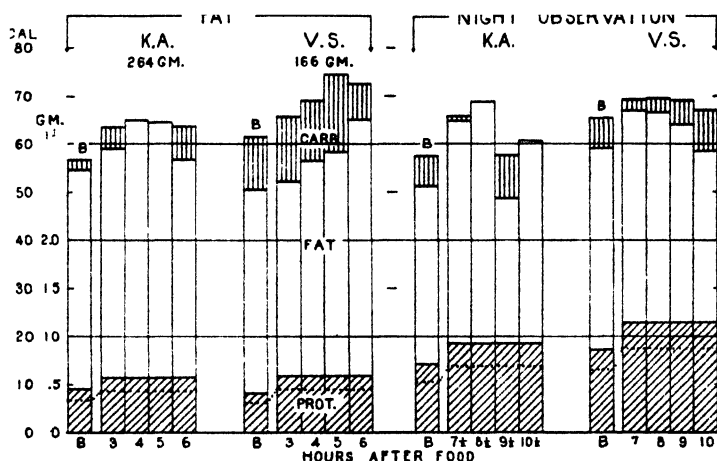


CHART III. The hourly heat production after fat meals and during the night. The dotted lines represent the gm. of urinary nitrogen per hour.

they found that 64 per cent of his energy was derived from protein in the 5th hour after the meal.

To summarize, the increased heat production for the 3rd, 4th, and 5th hours following meals of lean meat varied from 14.6 to 45.3 per cent of the basal heat output. The variation was due in part to the different amounts of meat eaten but the data suggest that the specific dynamic action of protein was greater at the end of a year on the meat diet.

Fat—After the period of the regular meat diet was completed the men took diets very high in fat and during this time one observation was made on each man after a large meal of fat (Chart

III). V. S. ate a meal of 40 per cent cream, butter, and beef fat, containing 166 gm. of fat, 4 gm. of protein, and 6 gm. of carbohydrate. His increased heat production represented 14.5 per cent of his basal energy output. He had received in this period 35 gm. of carbohydrate daily which accounted for the carbohydrate utilized in this observation. K. A. took 264 gm. of fat, 11 gm. of protein, and 13 gm. of carbohydrate furnished by 40 per cent cream and butter. His output of heat was 13.2 per cent above his basal level. K. A. took only 45 gm. of protein and no extra carbohydrate in the period preceding this observation.

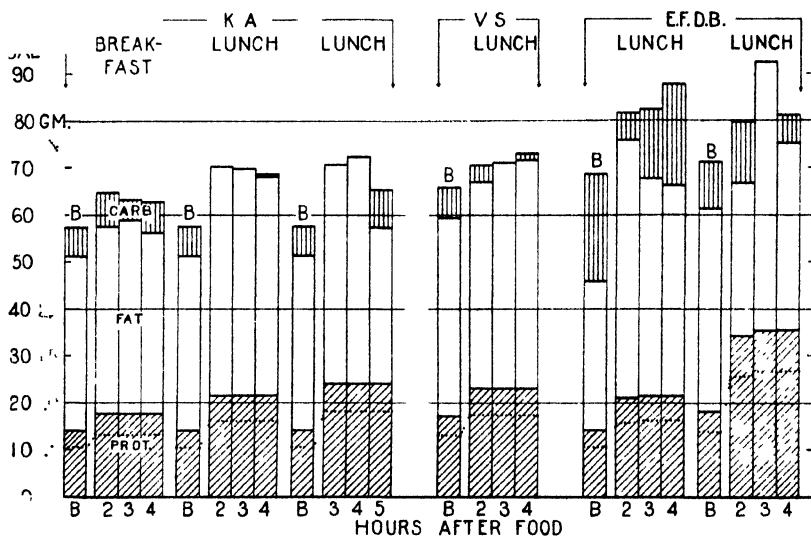


CHART IV. The hourly heat production following protein-fat meals. The first test on E. F. D. B. was made while he was taking a mixed diet. The others were made when the men were receiving meat exclusively. The dotted lines represent the gm. of urinary nitrogen per hour.

These figures were somewhat greater than those usually given for the specific dynamic action of fat but they represented findings under unusual conditions. Both men were excreting acetone bodies in their urine—V. S., approximately 1 gm. daily and K. A., 7 gm. If the specific dynamic action of fat is due to a plethora of metabolites as stated by Lusk (8), then a relatively high response could be expected as these men were receiving from 200 to 300 gm. of fat in their diets daily.

Lean Meat-Fat Combinations—The metabolism of the men, following mixed meals of lean and fat meat together, was studied eight times, once after breakfast, five times after lunch, and twice starting about midnight, 7 hours after the evening meal; V. S. was the subject twice, K. A., four times, and E.F.D.B., twice. These observations are summarized in Charts III and IV. All the tests except the first, with E.F.D.B., were made while the men were taking meat exclusively. They received from 150 to 200 gm. of lean meat (32.0 to 42.6 gm. of protein) and from 50 to 90 gm. of fat in each meal of the day of the test.

The maximum heat production occurred in the 3rd or 4th hour after the meal and the average for the 2nd to 4th hours was from 8.6 to 22.3 per cent greater than their basal metabolism. In the two tests at night the average increase for 4 hours was 4.8 per cent for V. S. and 10.5 per cent for K. A. The men slept about one-half of the time in these two observations.

Respiratory Quotients

The basal respiratory quotients during the meat and the high fat diets averaged 0.775 for V. S. in four observations and 0.760 for K. A. in eight determinations. The average basal quotient for the two men was 0.765. Following food the quotients were lower, V. S. in five experiments had a quotient of 0.756 and K. A. in seven observations, 0.742, while the average for both was 0.748.

The non-protein R.Q. was 0.751 when the men were in basal condition and 0.721 following food. The difference was due to the greater protein metabolism after meals as shown by the nitrogen in the urine.

When the hourly quotients obtained after meals were considered with regard to the time elapsed after taking the food, the average quotient for both men was 0.74 during the 2nd, 3rd, and 4th hours after the meal and 0.76 for the 5th and 6th hours. The influence of digestion with the production of an acid secretion in the stomach has been considered as a possible explanation for the slightly lower level of the R.Q. during the earlier hours after the meal. Dodds and his coworkers (9-11) found that the carbon dioxide content of alveolar air and blood rose during the 1st hour following meals and that it fell below the normal level in the 2nd and 3rd hours. The rise they associated with gastric digestion

and the fall with digestion in the duodenum. Chanutin (12) reported that the alkaline reserve of a dog, as measured by the carbon dioxide-combining power of the blood plasma, was materially increased after the ingestion of meat. No observations have been found in the literature where the carbon dioxide of alveolar air and blood, the gastric acidity, and the R.Q. have been determined at the same time. However, the studies reported suggested to us that a retention of carbon dioxide in the body occurred while hydrochloric acid was being secreted in the stomach and that this might last for 2, 3, or possibly 4 hours after a meal of lean meat accounting in part for the lower level of the R.Q. at this time. Then, later, as the acid was reabsorbed, carbon dioxide would be released, which would explain the higher level of the quotient in the 5th and 6th hours. Another explanation is the possibility that the storage of glucose during the time when the protein metabolism was at its height might have caused a slightly lower quotient at this time.

The constancy of the level of the R.Q. both in basal condition and after food, was striking. When the average for each experiment was taken, the levels for all observations fell between 0.72 and 0.78 during the time when the subjects were living on meat alone. This uniformity in the level of the R.Q. occurred in several men, previously reported (13), who derived nearly all their energy from protein and fat. A thorough discussion of the various factors which affect the R.Q. has recently been presented by Richardson (14).

Nature and Source of Foodstuffs Metabolized

The amount of protein metabolized during the observations in the calorimeter was calculated from the nitrogen in the urine and the division of the non-protein calories between fat and carbohydrate was determined from the modified Zuntz-Schumburg tables (Lusk 15). When the men were in basal condition they derived from 25 to 35 per cent of their energy from protein, from 60 to 70 per cent from fat, and from 5 to 10 per cent from carbohydrate. Following food the protein provided from 30 to 45 per cent (in one experiment with K. A. 63 per cent) of the energy output; fat, from 55 to 65 per cent; and carbohydrate, from 2 to 8 per cent.

The diet adequately provided all the protein and the fat utilized. It contained at most 50 calories of carbohydrate per day in the form of glycogen, which represented between 1 and 2 per cent of the energy intake. In the basal observations which were made 16 to 20 hours after the last meal, the calculations indicated that about 10 per cent of the metabolism (5 to 6 calories per hour) was obtained by the utilization of carbohydrate. In the determinations made after meals the men derived only 3 to 5 per cent of their energy from carbohydrate. The influence of the meals practically lasted through the entire 24 hours. This is shown by the fact that 10 hours after the evening meal the men had not reached the level of their basal heat output. Therefore with only 14 hours intervening between their supper and breakfast they were in basal condition not more than 2 or 3 hours in the whole day. The observations after meals of lean meat suggested that some of the glucose which was derived from protein was stored during the time when the protein was being utilized. This stored glucose may have been the carbohydrate which provided a small portion of the energy when the men were in basal condition.

In considering other possible sources of the carbohydrate which might be available during the basal observations, the influence of fecal nitrogen was studied. If the fecal nitrogen were included with the urinary nitrogen it would not change the findings by more than 0.5 per cent. Some might suspect that stolen food provided extra carbohydrate, but this is extremely unlikely as the levels were the same in all three subjects and they were under observation 24 hours daily during the intensive studies. Continued depletion of the glycogen of the body could not be a source as the R.Q. remained constant after the 1st week. A small error in the theoretical R.Q. for protein or for fat is not at all impossible. In spite of the excellent alcohol checks we doubt if any metabolic apparatus gives respiratory quotients closer than 0.01 or 0.02 and we realize that practically all work in respiratory metabolism may show an error of at least 1 per cent.

Assuming that the men were in the absorptive state 22 hours per day and in basal condition only 2 hours and assuming that the theoretical calculation and actual respiratory quotients are absolutely accurate, we had indications of oxidation of 34 gm. of carbohydrate per day in excess of the amount consumed in the

food. This is a small but significant discrepancy. Support for the suggestion that the theoretical R.Q. for fat may be higher than 0.707 under the special conditions of our observation was found in the work of Krzywanek (16) who noted that the elementary analysis of mutton fat resulted in an R.Q. of 0.721 and that the R.Q. of other animal fats varied from 0.710 to 0.723. As our subjects used mutton fat in considerable quantities, a recalculation of our data with a modified Zuntz-Schumburg table based on an R.Q. of fat as 0.721, reduced the discrepancy in the carbohydrate balance from 34 to 12 gm. per day. Such errors could be detected only in prolonged experiments of this kind and there are very few of them in the literature.

TABLE III
Comparison of Indirect and Direct Calorimetry

No. of subjects	No. of observations	Time <i>hrs.</i>	Calories		Variation of direct from indirect method	Nature of observations
			Indirect	Direct		
3	18	53	3208.50	3275.94	+2.1	Basal
2	6	24	1752.17	1689.58	-3.6	Meat
2	2	8	539.40	520.22	-3.6	Fat
2	2	8	529.30	532.05	+0.5	Night runs
3	6	18	1326.02	1305.53	-1.5	Mixed meals
3	34	111	7355.39	7323.32	-0.4	Total

Some might on first thought consider this discrepancy as evidence for the formation of carbohydrate from fat. A little calculation, however, will show that the formation of carbohydrate from fat would lower the theoretical R.Q. thus leaving a higher proportion of the observed R.Q. to be accounted for by carbohydrate from some other source. This would bring us back to just where we started. The only way for the adherents of this theory to prove their point from our observations would be to show that our subjects formed carbohydrate from fat while they were outside the calorimeter and then began to oxidize this carbohydrate as soon as they were in the respiration apparatus. At first this seemed a possibility but we felt that we ruled it out by observing the subjects at all hours of the day and night, awake and asleep,

fasting and after meals. It was unfortunate that we could not observe their metabolism during exercise but we are not acquainted with any work which shows that carbohydrate derived from fat is stored during exercise.

Comparison of the Indirect and the Direct Methods of Heat Measurement

The heat production, as measured by direct calorimetry for thirty-four observations with a total of 111 hours, was 0.4 per cent less than that determined by the indirect method. These comparisons made for groups of experiments of the same nature are presented in the Table III. They indicate that there was no significant error in the technique of these two calculations even in the presence of a continued mild ketosis.

SUMMARY AND CONCLUSIONS

1. The respiratory metabolism of three men was studied before and after meals during the day and night in a calorimeter which was demonstrated to be as accurate as any respiratory apparatus yet employed. Methods of direct and indirect calorimetry agreed within 0.4 per cent. When the men were taking the exclusive meat diets, the R.Q. remained between 0.72 and 0.78 at all times during the 24 hours they were observed.

2. The basal metabolic rates for two men varied between 80 and 91 per cent of normal. The meat diet caused no significant elevation of their metabolism.

3. The excretion of nitrogen in the urine varied from 0.5 to 0.8 gm. per hour when the men were in basal condition. For three of the tests following lean meat it was between 0.95 and 1.13 gm. hourly and in the last 2 hours of one test it reached the high rate of 2.12 gm. per hour.

4. Lean meat in meals of 300 to 500 gm. induced extra heat from the 3rd to 5th hours inclusive which ranged from 14.6 to 25.0 per cent of the basal heat production. In one test the increase was 45.3 per cent and the subject derived 63 per cent of his energy from protein.

5. With use of the standard figures for the theoretical R.Q. of protein and fat, the calculated foodstuffs oxidized did not agree exactly with the composition of the diet. The standard calcula-

tions indicated that approximately 34 gm. of carbohydrate were utilized per day in excess of that contained in the diet. This discrepancy may be due to small errors in the theoretical respiratory quotients for fat or protein or both. Our subjects derived about 75 per cent of the energy from beef or mutton fat. A recalculation, with a respiratory quotient for fat of 0.721, reduced the discrepancy in the carbohydrate balance to 12 gm. per day. It is evident that a significant error in the metabolic balances for carbohydrate may be possible, even with the best of our modern technique, if we employ the customary standard methods of calculation.

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THE OXIDATION CATALYSIS OF CRYSTALLINE GLUTATHIONE WITH PARTICULAR REFERENCE TO COPPER

By CARL VOEGTLIN, J. M. JOHNSON, AND SANFORD M. ROSENTHAL

(From the Division of Pharmacology, National Institute of Health, United States Public Health Service, Washington)

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In a recent paper (1) we have shown that crystalline glutathione, prepared according to the method of Hopkins (2) or of Kendall, McKenzie, and Mason (3), is susceptible to oxidation catalysis by minute amounts of copper salts. Under the same conditions iron salts do not exert a catalytic action. We were able to confirm the conclusion of Meldrum and Dixon (4) that the action of hematin upon glutathione is of a low order, in fact so low that it cannot be regarded as catalytic in the ordinary meaning of this term. Evidence was also obtained which suggested that the relatively slow rate of oxygen consumption of crystalline glutathione, dissolved in phosphate or pyrophosphate buffer within the physiological pH range, is due to incomplete removal of traces of copper from the crystalline products. We were unable to confirm Meldrum and Dixon's (4) claim that the heavy metal catalysis of crystalline glutathione is conditioned by the presence of an active iron-cysteine complex. It should be mentioned that our experiments, in contradistinction to those of Meldrum and Dixon, were carried out without the use of tissue residues or washed tissues, which we believe considerably complicate conditions, and for this reason are not well suited for a purely physical-chemical research.

The purpose of this paper is to present further evidence bearing upon the oxidation of crystalline glutathione. The following problems will be discussed. (1) Is the catalytic action of copper unique or are there other metallic and non-metallic compounds having a similar action? (2) Is it possible to remove the minute traces of copper in crystalline glutathione, so as further to decrease

the oxygen consumption of solutions made from this specially prepared material?

EXPERIMENTAL

The oxygen consumption was measured in the same Barcroft-Warburg micro respiration apparatus used in the previous work. The respiration vessels were provided with a side arm, which permitted the addition of solutions of chemicals to the glutathione solutions in the main compartment. The final volume of fluid in the main compartment was in all experiments 2.6 cc. The temperature was 37.6° and air was used as a source of oxygen. The respiration vessels, pipettes, and other glassware were freed from heavy metal impurities by treatment with chromic acid cleaning fluid, followed by thorough rinsing with water twice distilled in a Pyrex glass apparatus. This water was also used for preparing the solutions.

The glutathione used in this work was made according to Pirie's (5) modification of the Hopkins method, with exception of the omission of alcohol in the crystallization. The description of a method for the preparation of crystalline glutathione having an exceedingly low copper content and showing a very low oxygen consumption will be described later. The cysteine hydrochloride was prepared according to the method of Warburg (6) for the purpose of removing catalytic metals. This cysteine showed a very low oxygen consumption in phosphate buffer of pH 7.27. The metal salts and compounds were of the highest purity obtainable from commercial sources. The cobaltous chloride and selenic acid were especially purified by us.

Comparison of Action of Various Metallic and Non-Metallic Compounds with Action of Copper Salts—The glutathione used was made according to Pirie, and 15 mg. were added to the phosphate buffer (pH 7.27) in each of the respiration vessels. The solutions of the compounds, whose action was to be ascertained, were placed in the side arms. After the temperature of the equipment had adjusted itself to the constant temperature water bath and the stop-cocks had been closed, the solutions of the compounds were added to the glutathione in the main compartments and the manometric readings were begun.

The oxygen consumption of the control glutathione solution in

the different experiments varied little from 34 c.mm. for the 1st hour, indicating that this glutathione contained a trace of catalytic copper, as was to be expected from our previous work.

TABLE I
Effect of Various Metal Compounds upon Rate of Oxidation of Crystalline Glutathione. Phosphate Buffer, pH 7.27.

Metal compound	Metal added	Mol. weight of SHG	Effect on rate of oxidation
		Atomic weight of metal	
	mg.		
$\text{CuSO}_4 \cdot 4\text{NH}_3 \cdot \text{H}_2\text{O}$	0.01	1:0.00322	Acceleration 900 per cent
	0.0001	1:0.0000322	" 100 " "
H_2SeO_3	0.12	1:0.031	" 1300 " "
H_2TeO_3	0.05	1:0.008	" 500 " "
PdCl_2	0.1	1:0.0192	" 50 " "
$\text{AuCl}_3 \cdot 2\text{H}_2\text{O}$	0.1	1:0.0104	" 47 " "
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.052	1:0.0180	" for 20 min.
$\text{Fe}(\text{SO}_4)_3(\text{NH}_4)_2 \cdot \text{SO}_4$	0.1	1:0.0367	No effect
$\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$	0.1	1:0.037	" "
$\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$	0.1	1:0.035	" "
$\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$	0.1	1:0.0173	" "
$\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 2\text{H}_2\text{O}$	1.0	1:0.100	" "
As_2O_3	1.0	1:0.273	" "
CeCl_2	0.1	1:0.0146	" "
H_2OsO_4	1.0	1:0.107	" "
HgCl_2	0.1	1:0.010	" "
$\text{HPtCl}_6 \cdot 6\text{H}_2\text{O}$	0.1	1:0.0105	" "
$\text{K}_2\text{Cr}_2\text{O}_7$	0.1	1:0.039	" "
AgSO_4	1.0	1:0.190	Inhibition, complete
	0.1	1:0.019	" 88 per cent
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	1.0	1:0.3135	" complete
	0.1	1:0.0313	" 70 per cent
$\text{Cd}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 3\text{H}_2\text{O}$	0.1	1:0.0182	" 55 " "
$\text{Bi}(\text{NH}_4)$ tartrate	1.0	1:0.1	" 47 " "
	0.1	1:0.01	" 15 " "
$(\text{SbO})\text{K}$ tartrate	1.0	1:0.17	" 70 " "
	0.1	1:0.017	No effect

The results are summarized in Table I. The second column contains the data referring to the amount of metal (in mg.) which was added to 15 mg. of glutathione. The third column gives the

relation of the molecular weight of glutathione to the atomic weight of the metal in the actual ratio after mixing the two solutions. In the last column there is given the effect of the metal compound on the rate of oxidation of the glutathione in the first part of the experiments, during which the oxygen consumption was a linear function of time. The compounds are arranged in three groups. The first of these contains compounds which cause a marked acceleration. The most active of these is copper. The second group includes compounds which show no influence on the rate of oxida-

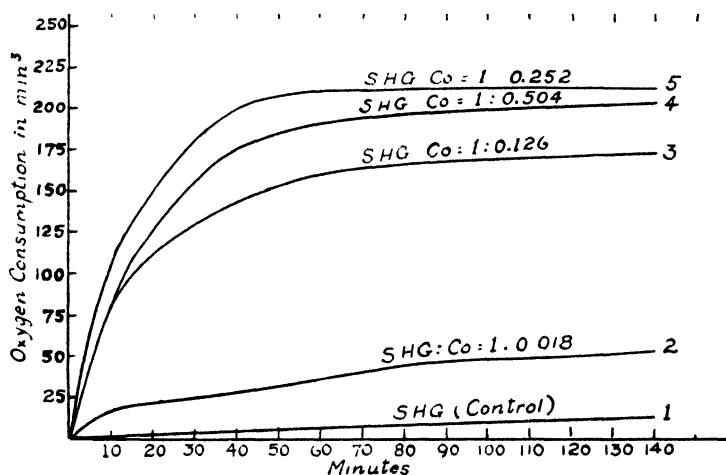


FIG. 1. Effect of cobaltous chloride (purified) upon the oxygen consumption of 15.3 mg. of crystalline glutathione (Lot 5945). Phosphate buffer pH 7.27, temperature 37.6°.

tion. The third group contains compounds which, in the amounts used, decreased the rate of oxidation.

The effect of cobaltous chloride is further illustrated by Fig. 1. This experiment was carried out by using a specially prepared glutathione (Lot 5945) containing an exceedingly small amount of copper. The commercial cobaltous chloride was purified by precipitation with a large excess of copper-free ammonia, filtration of the hydroxide, and reversion into the chloride by specially distilled hydrochloric acid. Compared with the catalytic action of copper, the activity of cobalt is of a much lower order. The small-

est amount of cobalt used (Curve 2) produced a rapid increase of oxygen uptake during the first 10 minutes. The rate then declined somewhat below that of the control. If the cobalt concentration is increased the rate of oxidation increases up to an optimum range (Curve 5) and then slightly decreases (Curve 4). In every experiment we found that the addition of cobalt to the glutathione in buffer solution immediately gives rise to a brown coloration. This brown complex is produced only in the presence of oxygen. If the glutathione in buffer solution is placed in a Thunberg tube pro-

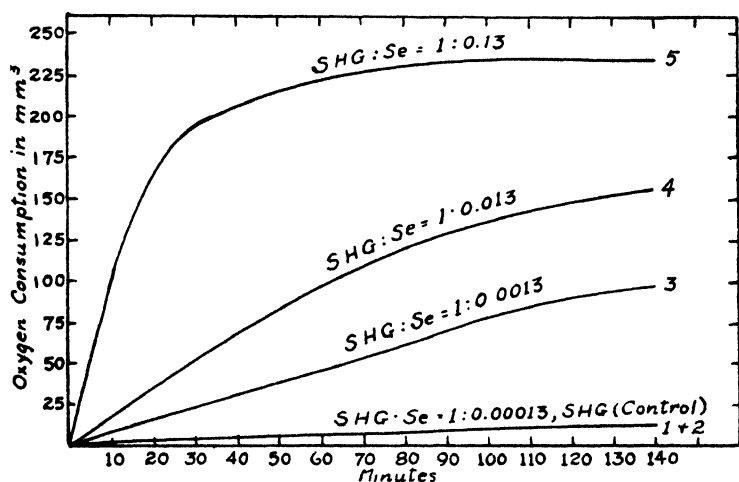


FIG. 2. Effect of selenic acid upon the oxygen consumption of 15 mg. of crystalline glutathione (Lot 5945). Phosphate buffer pH 7.27, temperature 37.6°.

vided with a loose inner tube containing the cobalt solution and the solutions are then evacuated by an efficient pump, the two solutions can be mixed without the brown complex being formed even after several days standing. If air is admitted the mixture turns brown. Michaelis and Barron (7) have found that cysteine and cobalt also react in the presence of oxygen with the formation of a brown complex, which they consider as being cobaltic cysteine produced by rapid oxidation of the cobaltous cysteine first formed. Part of the initial increase in rate of oxidation of glutathione following the addition of cobaltous chloride may be caused by a similar

reaction. However, it would be premature to postulate a definite reaction mechanism on the basis of the present evidence. The reaction mechanism of cobalt and cysteine has recently been studied by Kendall and Holst (8).

In Fig. 2 there is shown an experiment with sodium selenate, in place of the selenite experiment which is included in the summary table (Table I). These results show that selenium in the form of selenate is more active than cobalt in the oxidation of glutathione. Both selenite and selenate produce a brownish color when added to

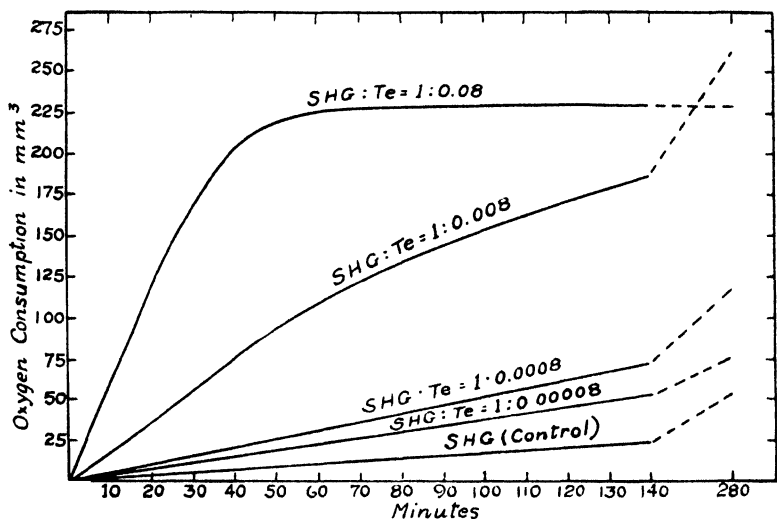


FIG. 3. Effect of telluric acid upon the oxygen consumption of 15 mg. of crystalline glutathione (Lot 5945). Phosphate buffer pH 7.27, temperature 37.6°.

glutathione solutions, and if added in sufficient amounts yield solutions which no longer give the nitroprusside test. It is well known that selenious and selenic acids and their salts are easily reduced by a variety of reducing agents. It is reasonable to suppose, therefore, that glutathione is a reductant, being itself oxidized while the selenium salts are reduced.

Fig. 3 illustrates an experiment dealing with the action of tellurate and glutathione and indicates that this compound is slightly more effective than the corresponding selenium compound. Here

also, when the larger amounts are employed, a brown color is developed and the same considerations apply as those just stated for selenic acid.

The ineffectiveness of the metal compounds of the second group cannot be attributed to lack of solubility (precipitate formation) in the glutathione in buffer solution. Under the conditions of these experiments only the iron, tin, and lead salts produced a visible turbidity. The other metal compounds either form soluble complexes with glutathione or do not enter into chemical reaction. The addition of $K_2Cr_2O_7$ to the glutathione solution produced a green color.

The metals of the third group, when added in relatively small amounts, produce a retardation in the oxygen consumption without causing a visible turbidity. In the case of bismuth the glutathione solution turns yellow. Some of these metals undoubtedly form complexes with glutathione, as will be shown later. It is possible that this complex formation is related in some way or other to the inhibition in the rate of oxidation.

Removal of Copper from Crystalline Glutathione—The crystalline glutathione prepared by means of the Hopkins method was shown by us (1) to yield products containing about 0.013 mg. of Cu per 1000 mg. of glutathione. This value may vary considerably according to the conditions, but at all events is sufficient to explain the oxygen consumption of glutathione on the basis of a copper catalysis. It is not at all surprising that glutathione should contain traces of copper, as glutathione is isolated in the form of a crystalline copper complex. This latter is then decomposed by H_2S , the copper sulfide is filtered off, and the filtrate is allowed to crystallize. In the previous paper (1) we confirmed Meldrum and Dixon's (4) observation that kaolin treatment of a *dilute* glutathione solution removes most of the catalytic metal. But we found this method unsatisfactory for the purification of a considerable amount of glutathione, because dilute solutions and excessive quantities of kaolin were needed.

In the preceding experiments it was shown that cadmium and silver salts do not act as catalyst in the oxidation of glutathione. It was therefore decided to precipitate crystalline glutathione by means of these metal salts, to separate the metal complexes, and to decompose them with H_2S . The filtrates were then allowed to crystallize. The procedure was the following.

Cadmium Method—1 gm. of crystalline glutathione, prepared according to Pirie, and containing 10.76 per cent S and 13.67 per cent N, was dissolved in about 10 cc. of glass-distilled water. To this solution was added cadmium acetate, dissolved in glass-distilled water, until maximum precipitation. An excess of the reagent has a tendency to redissolve part of the precipitate. The white amorphous precipitate was centrifuged off, washed several times with water, and finally decomposed with H_2S . The filtrate from the cadmium sulfide was treated for a short time with a current of pure air to remove H_2S , and set aside for crystallization in a vacuum desiccator over P_2O_5 . The crystals were dried to constant weight. Yield 0.7 gm.

Silver Method—2 gm. of crystalline glutathione were dissolved in about 20 cc. of glass-distilled water and precipitated with an aqueous solution of silver acetate. The white precipitate was filtered off, washed repeatedly with glass-distilled water, finally suspended in water, and decomposed with H_2S . The filtrate from Ag_2S was concentrated in a vacuum at room temperature and allowed to crystallize. The crystalline mass was filtered off and dried to constant weight in a vacuum desiccator over P_2O_5 . Yield 1.425 gm. (Lot 5945). Lot 5886 was prepared by silver sulfate in place of silver acetate. This glutathione on analysis gave 10.71 per cent S and 13.55 per cent N. Iodine titration in alcoholic solution indicated that over 96 per cent of the sulfur was in the reduced form.

The application of the recent modification of the Sullivan test (9) to our samples of crystalline glutathione, whether prepared by Pirie's method, or by means of cadmium, or silver precipitation, in no instance gave an indication of the presence of the least amount of cysteine or other impurity reacting with the naphthoquinone reagent.

Fig. 4 shows the exceedingly low oxygen uptake of glutathione Lot 5886 (silver method) dissolved in pyrophosphate buffer at pH 7.60 and illustrates furthermore the powerful catalytic effect of copper in minute amounts. Iodine titration of this sample of glutathione indicated that all of the sulfur was in the reduced state.

Fig. 5 shows a similar experiment indicating the ineffectiveness of iron as a catalyst, either when added alone or together with a small amount of pure cysteine. The increase in oxygen consump-

tion of the combination glutathione, cysteine, and iron (Curve 3) over glutathione (Curve 2) is fully accounted for by the oxygen consumed by this amount of cysteine. The theoretical O_2 uptake of cysteine is indicated by the vertical bar on the right-hand side of Fig. 5.

We found that copper alanine and copper thioglucose¹ have the same catalytic activity as the same amount of copper in the form of inorganic salts.

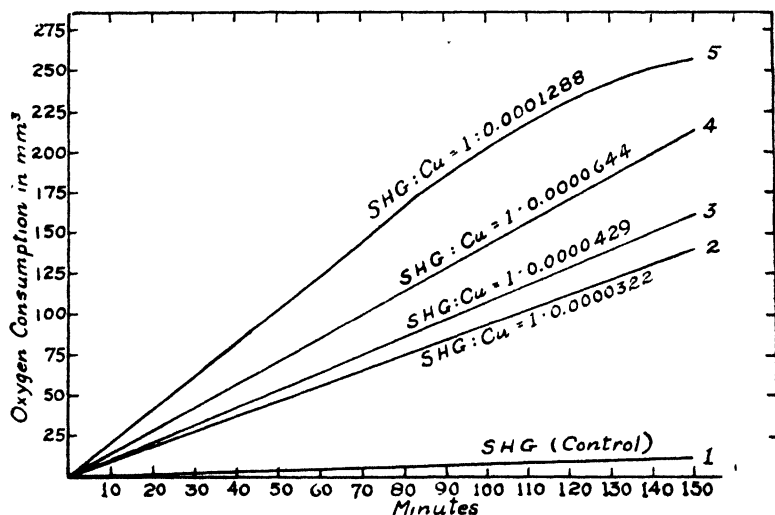


FIG. 4. Catalytic action of copper sodium citrate upon the oxidation of 15 mg. of crystalline glutathione (Lot 5886). Pyrophosphate buffer pH 7.60, temperature 37.6°.

Experiments carried out for the purpose of estimating the *total* oxygen consumption of crystalline glutathione in the presence of catalytic copper in pyrophosphate buffer of pH 7.6 showed that the theoretical amount of oxygen was required for the conversion of reduced glutathione into the disulfide. In other words, the oxidation of SH glutathione to S-S glutathione by means of copper and O_2 requires the same amount of O_2 as the oxidation of the same number of molecules of cysteine to cystine. As soon as the

¹ These two substances were prepared by Dr. M. E. Maver in this laboratory.

oxygen consumption of the glutathione solutions ceases, the solutions no longer give the nitroprusside test, a further indication of the completion of the oxidation.

As many oxidations are accompanied by the formation of hydrogen peroxide, we decided to test this possibility in the case of the copper catalysis of crystalline glutathione.

100 mg. of glutathione were dissolved in 20 cc. of phosphate

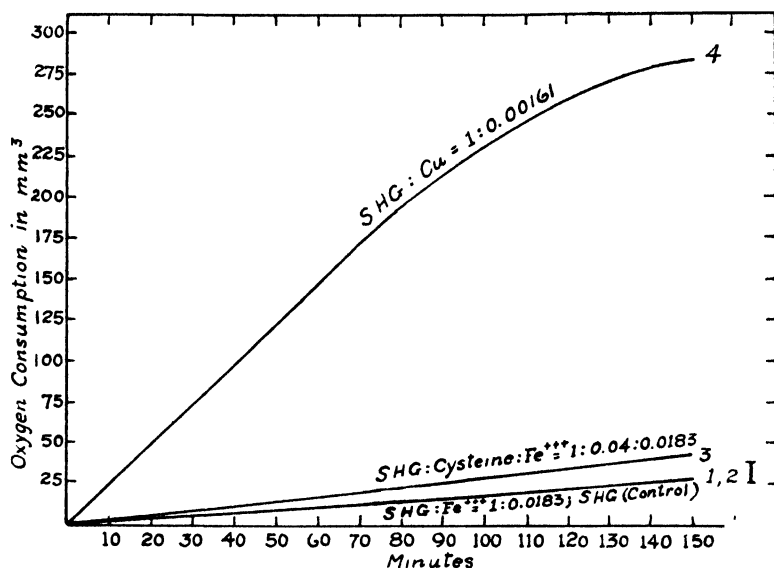


FIG. 5. Comparison of the effects of copper and iron salts upon the oxygen consumption of 15 mg. of crystalline glutathione (silver method). Showing the absence of a catalytic action of iron salt alone or in combination with small amounts of cysteine. Phosphate buffer pH 7.27, temperature 37.6°. The theoretical O_2 uptake of cysteine is indicated by the vertical bar at the right-hand side of the figure.

buffer (pH 7.27) and 0.025 mg. of copper in the form of copper ammonium sulfate was added. A current of oxygen was passed through the solution until the nitroprusside test became negative. 10 cc., representing 50 mg. of glutathione, were treated with a dilute solution of titanous chloride and the other 10 cc. were treated with a freshly prepared suspension of cerous hydroxide in water. A control solution, containing 0.075 mg. of H_2O_2 in 10 cc. of phos-

phate buffer (pH 7.27) gave a deep yellow color with both reagents, whereas the glutathione solutions showed only the faintest yellow color. If, therefore, H_2O_2 is formed in the oxidation of glutathione under these conditions, the amount would be far less than 0.075 mg. of H_2O_2 in 10 cc. of solution, or less than 1 part in 133,333. It was furthermore shown that the addition of 0.01 mg. of cerium as cerous chloride to 15 mg. of glutathione plus 0.001 mg. of Cu dissolved in phosphate buffer pH 7.27 did not affect the rate of oxygen consumption of glutathione caused by the catalytic action of copper. If, therefore, H_2O_2 is produced during the copper catalysis of glutathione, the amount must be exceedingly small.

Experiments were carried out to estimate the small traces of copper present in the glutathione which had been prepared by means of cadmium or silver precipitation. Weighed samples of these preparations were ashed in quartz crucibles in an electric furnace. The crucibles were then treated with a few drops of glass-distilled HCl. This solution was carefully neutralized with pure NaOH solution. The catalytic effect of the solution on the oxidation of the same glutathione which was used for ashing, was then determined in comparison with the action of known amounts of copper. The experiments were carried out in pyrophosphate buffer at pH 7.60, with the same technique as was used in the experiments described in the preceding paper (1). Thus it was shown that Lot 5885, which had been prepared by the cadmium method, contained 0.0025 mg. of Cu per gm.; and Lot 5886, prepared by the silver method, contained 0.00125 mg. of Cu per gm. It will be recalled that we had found (1) that crystalline glutathione prepared by the Pirie method contained 0.013 mg. of Cu per gm. Thus it is seen that cadmium precipitation reduced the copper content to approximately one-sixth and silver precipitation to one-tenth of the copper content of the Pirie preparation. These approximate figures are in harmony with the reduction in the rate of oxygen consumption of these specially prepared glutathione samples, as compared with the oxygen consumption of glutathione prepared with the Hopkins method or the Pirie modification.

Action of Iron and Copper on the Oxidation of Glutathione Which Has Previously Been Heated in Vacuo—Hopkins (2) has shown that long continued boiling of an aqueous solution of glutathione causes partial hydrolysis with the separation of glutamic acid (or pyr-

rolidonecarboxylic acid) and the formation of the diketopiperazine of glycylcysteine. Kendall, Mason, and McKenzie (10) and Mason (11) later showed that this decomposition takes place by prolonged incubation even at 37° . They consider that cysteinylglycine is formed, which is in harmony with present information concerning the constitution of glutathione. The evidence, therefore, indicates that glutathione is relatively unstable and easily broken down. Hence it was of interest to study the action of iron

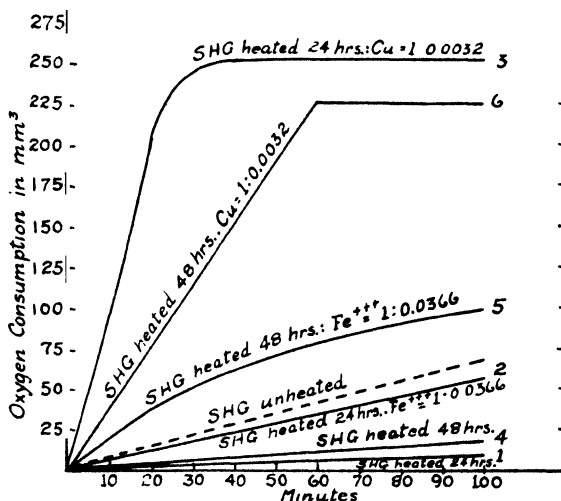


FIG. 6. Effect of copper or iron ammonium sulfate upon the oxygen consumption of solutions of crystalline glutathione (Pirie method), which had been heated in a vacuum to 37° for 24 and 48 hours respectively. Long continued heating of aqueous glutathione solutions causes partial decomposition and renders such solutions susceptible to the action of iron salts.

and copper salts upon the oxidation of glutathione which had been subjected to moderate heating. For this purpose 120 mg. of crystalline glutathione (Lot 5835) prepared by the Pirie method, were dissolved in 3.2 cc. of glass-distilled water. This solution was divided into 2 equal parts, and placed in two carefully cleaned Thunberg tubes. The tubes were then evacuated by a high vacuum pump and placed in an incubator at 37.5° . One of these tubes was opened after 24 hours and aliquots of 15 mg. of glutathione were placed in a series of respiration vessels containing

phosphate buffer, pH 7.27. The action of iron and copper (as ammonium sulfates) is shown in Fig. 6, in Curves 1, 2, and 3. For comparison the oxygen uptake of 15 mg. of the same glutathione in the same phosphate buffer is given (broken line). It will be seen that the rate of oxygen consumption of the heated glutathione solution is considerably less than that of the freshly prepared solution. The reason for this difference is not evident. The heated solution again shows the pronounced catalytic effect of copper, but also shows that iron induces an increased rate of oxidation. Under exactly the same conditions the contents of the second Thumberg tube were tested after 48 hours of incubation, as shown by Curves 4, 5, and 6. Copper again is highly active and the action of iron is further increased. We conclude, therefore, that *long continued incubation in vacuo* of solutions of crystalline glutathione results in chemical changes which render such solutions susceptible to the action of iron salts, whereas the oxygen consumption of freshly prepared solutions is not influenced by the same or even larger amounts of iron salts.

It is interesting to note that the Sullivan test (9) of freshly prepared solutions of our various crystalline glutathione samples has never indicated the presence of impurities. Nor do solutions of glutathione which have been oxidized by traces of copper give a positive test. However, we could easily show that the heated samples, before and after treatment with copper or iron salts in the Warburg apparatus, give uniformly a pronounced purple-red color, which cannot be matched with the red color produced by cysteine. Johnson and Voegtlin (12) observed similar purple color tests with some of their samples of amorphous glutathione. Recently Mason (11) found that an impure sample of cysteinyl-glycine obtained by heating crystalline glutathione also gives a purple-red color with the naphthoquinone reagent. We therefore prepared a small amount of the anhydride of cysteinyl-glycine from a crystalline sample of the corresponding disulfide. The latter was dissolved in a small amount of hot water and reduced according to the directions given by Hopkins (2). Special care was taken to prevent contamination with traces of copper by using special reagents, glass-distilled water, and cleaned glassware. The cysteinyl-glycine anhydride was thus obtained in crystals melting at 204° (uncorrected) and giving a purple-red color with the Sul-

livan test, indistinguishable from the color produced by heated glutathione solutions.

A single experiment was performed to study the oxidation of this compound in phosphate buffer at pH 7.27. 4 mg. of the substance consumed only 5 c.mm. of O_2 in 150 minutes and the O_2 uptake was not influenced by the addition of 0.1 mg. of Fe. On the other hand 0.001 mg. of Cu increased the rate of oxidation 800 per cent in the same period. Another experiment showed that the addition to 15 mg. of crystalline glutathione in phosphate buffer of 0.2 mg. of cysteinyl-glycine anhydride plus 0.1 mg. of Fe did not cause an increased rate of oxygen consumption. It may be concluded, therefore, that under the conditions of these experiments, both crystalline glutathione and cysteinyl-glycine anhydride are susceptible to copper catalysis, but are unaffected by iron.

Complexes of Crystalline Glutathione with Silver, Cadmium, Mercury, and Arsenic—In view of the great tendency of sulfhydryl compounds toward heavy metal complex formation, and in view of the results described in these papers concerning the influence of certain metal compounds on the rate of oxidation of glutathione, it seemed of interest to attempt the preparation of such metal complexes in pure form. So far the only crystalline compound of this kind is the crystalline copper-glutathione of Hopkins, which contained 1 atom of copper attached to the sulfur of the glutathione molecule. We prepared the following compounds.

Silver-Glutathione—The preparation of this complex has already been described. It was separated in the dark room, thoroughly washed with water, and dried in a vacuum desiccator over P_2O_5 to constant weight. As first obtained the substance is a white amorphous solid which is only slightly soluble in water. Suspended in water it does not yield a positive nitroprusside test. This test, however, becomes strongly positive after treatment of the suspension with HCl, indicating that the Ag is firmly attached to S and is only split off by HCl. Analysis showed the compound to contain Ag 25.80, 25.79 per cent; S 7.51, 7.69 per cent; N 9.98, 9.95 per cent. Theory for $R \cdot SAg$, Ag 26.06 per cent; S 7.75 per cent; N 10.15 per cent.

Cadmium-Glutathione—The preparation of this complex has already been described in the preceding pages. The white freshly precipitated compound was centrifuged off, thoroughly washed

with water, and dried to constant weight. Yield 1 gm. from 1 gm. of glutathione. The compound is slightly soluble in water and gives a strong nitroprusside test. Analysis showed Cd 25.63, 25.83 per cent; S 7.63, 7.67 per cent; N 9.69, 9.79 per cent. Theory for $R\cdot SCdOH$, Cd 25.81 per cent; S 7.37 per cent; N 9.65 per cent.

Mercury Compound—1 gm. of crystalline glutathione was dissolved in water and precipitated with a solution of mercuric sulfate in water. The white, amorphous precipitate was centrifuged off, well washed with water, and dried to constant weight. Yield 1.4 gm. The nitroprusside test yields first a very faint pink, which turns immediately to brownish green and then into an intense permanent olive-green. Analysis showed Hg 48.98, 48.83 per cent; S 7.97, 8.07 per cent; N 5.68, 5.68 per cent. These figures come nearest to the empirical formula $R\cdot SHgOH\cdot HgSO_4$, which calls for Hg 48.90 per cent; S 7.81 per cent; N 5.12 per cent.

If mercuric acetate is used in place of mercuric sulfate, a product is obtained which yields analytical figures coming close to the formula $R\cdot SHgOH$. The composition of the mercury complex of glutathione, therefore, seems to vary according to the method of preparation.

Arsenic-Glutathione—The preparation of this complex presents considerable difficulties, as was pointed out by Johnson and Voegtlin (13). The compound is very easily soluble in water and must therefore be separated from alcoholic solution. 300 mg. of As_2O_3 were dissolved in about 150 cc. of absolute CH_3OH by heating to boiling and filtering from a small insoluble portion. 1.5 gm. of crystalline glutathione were dissolved in 15 cc. of H_2O and 150 cc. of hot absolute CH_3OH were added. The arsenic solution, while still warm, was added to the warm glutathione solution and the cloudy mixture was kept on the water bath for a short time to cause coagulation of the heavy white precipitate. This was filtered off without delay, washed well with absolute CH_3OH , and dried in a vacuum desiccator over P_2O_5 and paraffin to constant weight. Yield 0.7 gm. from 1 gm. of glutathione. The product is a white, amorphous powder, very easily soluble in water, and gives a strong nitroprusside test. This would indicate that the linkage between S and As is easily broken, as in the case of the crystalline cysteine arsenic derivatives (13). The product gave the following analytical results; As 7.40, 7.36 per cent; S 9.66, 9.52 per cent; N

12.19, 12.11 per cent. Theory for $\text{As}(\text{SR})_3$ As 7.55 per cent; S 9.69 per cent; N 12.69 per cent. In order to exclude the possibility of the mere formation of a mixture of the two components, i.e. glutathione and As_2O_3 being thrown out of solution, we intentionally used 1.8 times as much As_2O_3 as called for by the theory. The product obtained can therefore hardly be a mixture of uncombined reagents. It is of course always difficult to place complete confidence in the analytical results of amorphous compounds which cannot be obtained in crystalline form. Nevertheless these products appear to be definite chemical individuals.

DISCUSSION

Of the heavy metal salts so far studied with regard to their action on the oxidation of crystalline glutathione only copper, palladium, and gold cause an acceleration. Copper is by far the most effective catalyst. The relatively slight effect of palladium and gold may be due to the presence of traces of copper in the salts employed. Cobalt also accelerates the oxidation, but is of a lower order of activity. Selenium and tellurium (non-metals) are decidedly less effective than copper, but much more effective than gold and palladium. It is astonishing that iron and manganese are inactive as these metals are very effective catalysts in the oxidation of cysteine. There is obviously no consistent correlation between the order of these elements in the electromotive series and their action on the oxidation of glutathione in the presence of molecular oxygen. The only thing that can be said is that the active compounds without exception are compounds in which the active element can change its valence. This may be one of the factors in this oxidation catalysis, but it is obviously not the only one.

We have succeeded in removing all but infinitesimal traces of copper from crystalline glutathione and have shown that such preparations exhibit an extremely low oxygen consumption in solutions within the physiological pH range. None of our glutathione samples has given any indication of the presence of organic impurities; they are uniformly crystalline and do not react with the recent modification of the Sullivan reagents. We have furthermore shown that the addition of small amounts of pure cysteine plus iron salts to glutathione solutions has no influence on

the rate of oxidation of glutathione. This indicates that the oxidation of this substance is not dependent on the presence of a catalytically active iron-cysteine complex, but rather that the oxidation of glutathione is a copper catalysis. We have no reason to doubt that crystalline glutathione, if properly prepared without the use of heat and hydrolyzing agents, is a pure substance, in the same sense that cysteine hydrochloride can be prepared in pure form. The main difficulty in the preparation of pure glutathione is the removal of traces of copper, and we believe we have succeeded in this almost as well as Warburg in the case of cysteine free from heavy metals. In fact, we have found such specially prepared glutathione quite satisfactory for the quantitative estimation of minute amounts (0.0001 mg.) of copper. It would seem that glutathione is more satisfactory for this purpose than Warburg's cysteine method, for the reason that other heavy metals of physiological interest, *i.e.* iron and manganese, do not accelerate the oxidation of glutathione. We would suggest using a pyrophosphate buffer of about pH 7.6 for such copper estimations, as the copper effect is somewhat greater in pyrophosphate than in phosphate buffer.

We have found no evidence indicating that the oxidation of glutathione by copper is accompanied by the formation of hydrogen peroxide. The mechanism of this oxidation may be tentatively postulated as a reaction of cupric ion with the SH group with the formation of a cuprous glutathione complex, which is readily oxidized by molecular oxygen at slightly alkaline pH to the disulfide of glutathione and cupric ion. The continual progress of these reactions would thus account for the catalytic effect of the copper. Hopkins (2) has called attention to the ease of oxidation of the cuprous glutathione complex and our personal experience indicates that this complex is only stable in a strongly acid medium, and oxidizes as soon as the acid is removed.

Our experiments with glutathione solutions which have been incubated for a day or two in a vacuum clearly show that glutathione is broken down under these conditions and is now susceptible not only to copper but also to iron. We do not know the chemical nature of the iron-susceptible cleavage product. It is certainly not cysteinyl-glycine anhydride, as we have shown that this substance is not affected by iron, but curiously enough by

copper. Nor does the addition of a small amount of cysteinylglycine anhydride and iron to glutathione affect the rate of oxidation of the latter substance.

From the physiological view-point these results are of interest, because of the prevalence of glutathione and copper in most animal organisms. It is a curious fact that the other heavy metals which appear to be normal physiological components of tissues, as iron, manganese, cobalt, nickel, and zinc, do not exhibit the same degree of catalytic activity on glutathione as copper, or are inactive (Fe, Mn, Ni), or even inhibit (Zn) the oxidation. There are at least some indications of a physiological and pharmacological relationship between copper and glutathione. Voegtlin, Johnson, and Dyer (14) some years ago demonstrated the protective action of amorphous glutathione against the oligodynamic action of copper salts on *Spirogyra* and tadpoles and against the toxic action of copper on albino rats. We have recently confirmed these findings on rats by using crystalline glutathione. Furthermore, it is now possible to explain the recent observations of Rosenthal and Voegtlin (15) dealing with the rapid oxidation of crystalline glutathione, when added to blood serum. Blood serum contains quite sufficient copper to account for the oxidation of added glutathione. The action of copper on the "fixed" sulfhydryl groups of proteins and upon certain amino acids will be discussed in a subsequent communication.

SUMMARY AND CONCLUSIONS

1. The oxidation of crystalline glutathione in phosphate buffer of physiological pH is accelerated in the presence of relatively small amounts of the following substances: salts of copper, palladium, gold, and cobalt; selenate, selenite, and tellurite. Copper is by far the most effective catalyst.

Under the same experimental conditions the following substances do not influence the rate of oxidation of glutathione: salts of iron, manganese, nickel, tin, lead, cerium, mercury, platinum, chromium, osmic acid, and arsenite (in relatively low concentration).

Salts of silver, zinc, cadmium, bismuth, and antimony in suitable concentrations decrease the rate of oxidation of glutathione.

2. The so called autoxidation of crystalline glutathione, pre-

pared according to Hopkins, is due to the presence of minute traces of copper in this material.

Methods are described, which permit the preparation of crystalline glutathione of a much lower copper content. This product exhibits a correspondingly lower rate of oxygen consumption which is not influenced by the addition of a small amount of cysteine and iron salt, or a small amount of cysteinyl-glycine anhydride and iron salt.

3. Prolonged heating of aqueous glutathione solutions *in vacuo* at 37° causes decomposition. The addition of iron salts to this material mixed with phosphate buffer shows that the oxygen consumption is accelerated by iron salts.

The rate of oxidation of cysteinyl-glycine anhydride in phosphate buffer is greatly increased by copper salts, but not by iron salts.

4. Complexes of crystalline glutathione with silver, cadmium, mercury, and arsenic have been prepared.

5. A possible physiological relationship between copper and glutathione has been suggested.

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STUDIES ON THE CHEMICAL COMPOSITION OF BONE ASH

By SERGIUS MORGULIS

WITH THE TECHNICAL ASSISTANCE OF E. JANECEK

(From the Marine Biological Laboratory, Woods Hole, and the Department of Biochemistry, College of Medicine, University of Nebraska, Omaha)

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An understanding of the composition of bone is essential for the proper interpretation of the process of its formation in the living organism. It is obvious that definite physicochemical conditions are necessary to promote bone formation from components dissolved in the circulating fluid of the body. But it is still a moot question whether the physicochemical condition is the only factor or merely one of the essential factors determining the process. Viewed from the strictly physicochemical standpoint, the problem may be said to be in total confusion. Is bone formation simply a precipitation of insoluble salts and, if so, is this associated with a state of supersaturation of the serum with $\text{Ca}_3(\text{PO}_4)_2$, as is believed by some (3, 4), or with a state of undersaturation with CaHPO_4 , as is assumed by others (16, 17)? "All these conclusions," as is well pointed out by Roseberry, Hastings, and Morse, "may be independently correct and yet have no relation to the situation existing between serum and bone." Is the basic salt complex of bone a compound of the nature of $\text{Ca}[\{\text{Ca}_3(\text{PO}_4)_2\}_3]\text{CO}_3$ (Gassmann (2)) or of $\text{Ca}[\{\text{Ca}_3(\text{PO}_4)_2\}_6](\text{OH})_2$ (Klement (8, 9)) and, if so, does it possess a definite crystalline structure? On neither of these fundamental questions has a final answer yet been definitely obtained. The recent contribution of Robison and collaborators (12, 13) again brings out the significance of an enzyme mechanism for creating a condition of supersaturation in the matrix fluid necessary for calcification, and the possibility that phosphate esters may play an important rôle in the process of bone formation.

The contribution which this paper attempts to make to this controversial subject deals with a study of the balance of basic and acid equivalents in the bone ash. It is based upon an analysis of the mineral composition of the vertebræ in a variety of animal forms from fishes to mammals.

Analytical Results

The vertebræ, freed from soft tissue as nearly as possible, and defatted with boiling alcohol, were ashed by the process proposed by Gabriel (1) but with certain important modifications. The method consists in dissolving the entire organic matrix of bone by heating in a 3 per cent KOH solution in glycerol. We found it, however, essential to use a reagent previously freed from water by boiling and we carry out the ashing by heating the bone at about 250°. The bone thus leached out with alkaline glycerol reagent retains its morphological form perfectly, only the organic matter being destroyed and leaving a compact white mass of bone salts. Our procedure of washing the ashed bone is also an important departure from Gabriel's. The leached bone is boiled briskly with distilled water once or twice, depending on how much of the reagent adheres to the surface; it is then extracted several times in boiling 95 per cent alcohol, to remove as nearly as possible all the glycerol reagent, and is dried to constant weight at 110°. The bone in this condition can be easily reduced to a fine powder; indeed it crumbles when pressed between the fingers. The bone powder which may be snowy white or only slightly tinged, dissolves in hydrochloric acid without leaving any insoluble residue. The analyses were made on bone ash powders thus prepared. A weighed amount of powder dried to constant weight is dissolved in a measured quantity of standard HCl, calcium, magnesium, potassium, and phosphate being determined in aliquot portions of this solution by the usual procedures. Calcium was precipitated as ammonium oxalate, which was dissolved in sulfuric acid and titrated with 0.005 N KMnO_4 . The phosphates were determined by the Fiske-Subbarow colorimetric method. However, all the phosphate determinations were subsequently repeated by two other procedures; gravimetrically, by precipitating the phosphate as strychnine phosphomolybdate and also, volumetrically, by Fiske's method. Except for one or two instances the results

by all three methods checked perfectly and where a discrepancy occurred between the colorimetric determination and the others, the result of the gravimetric analysis was accepted. The analyses of the gravimetric method were accurate within ± 0.2 per cent. The magnesium was precipitated as MgNH_4PO_4 and the phosphate determined colorimetrically. Owing to the very small amounts of potassium present in the bone material the direct determination was not possible. We determined, therefore, the potassium by Kramer's method in a solution of known potassium content and in a similar sample to which an aliquot of the bone ash solution was added. The potassium of the bone ash was calculated from the difference between the two analyses. The carbonate was determined in weighed amounts of bone ash powder in a modified Van Slyke CO_2 apparatus according to Kramer's procedure (15).

In addition to the above analyses, an aliquot portion of the bone ash solutions was titrated with standard sodium hydroxide, methyl orange being used as an indicator, or, after boiling off the CO_2 , by titrating electrometrically to the pH of a KH_2PO_4 solution. The titrations by both procedures were the same and we, therefore, limited ourselves to the indicator titration only.

The first question which naturally presents itself is whether the treatment with the glycerol reagent does not alter the composition of the bone salts in some essential respect. We tested this point in two ways and feel satisfied that the procedure is unassailable. First, we analyzed a sample of tricalcium phosphate dissolved in hydrochloric acid, determining the Ca and P by the usual methods and from the results a Ca:P ratio of 1.92 was calculated for this material. Another sample of the same material was then subjected to the treatment with the alkaline glycerol, exactly as was done in the case of the bone material, the Ca and P analyses giving a Ca:P ratio of 1.92, just as for the untreated salt. Secondly, we analyzed untreated powdered bone and a sample of the same bone which had been leached out by the glycerol reagent. The results of the analyses of the two samples are recorded in Table I from which it is obvious that, correcting for the organic content of the untreated bone, boiling with the alkaline glycerol, and subsequent extraction with boiling alcohol does not affect the composition of the mineral components of the bones. The values for Ca and P are perhaps somewhat higher as determined on the bone ash

material than on the original unashed whole bone. However, the untreated bone powder was extracted with trichloroacetic acid according to Kramer's method of bone analysis (15), and this may account for the small differences. It is possible, of course, that the calcium was not completely extracted and, as far as the phosphate determination is concerned, the trichloroacetic acid extract of bone is decidedly less favorable than the bone ash solution for colorimetric analysis, the color in the former instance being greenish blue and difficult to match, whereas in the latter instance a beautiful pure blue color develops.

The results of our analytical study are presented in Tables II and III, in which the elementary composition of the ash and the balance of acid and basic equivalents, respectively, are given. In calculating the bone salt composition from the elementary

TABLE I

	Ash	Whole bone (50.7 per cent organic matter)	Ratio
	<i>per cent</i>	<i>per cent</i>	
Ca	32.90	15.89	1.02
P	15.75	7.68	1.01
CO ₂	2.32	1.17	0.98
CaCO ₃	5.28	2.66	0.98
Ca ₃ (PO ₄) ₂	79.55	38.65	1.01

analysis certain assumptions have been made. First, that the magnesium in the bone ash is present entirely in the form of Mg₃(PO₄)₂; secondly, that the predominant part of the potassium is in the form of KOH. Of course, some of the magnesium may be and probably is found as carbonate, but it does not affect the calculation materially even if the opposite assumption were made; namely, that all the magnesium in the bone ash is in the form of MgCO₃. As regards the potassium, although it is found in bone tissue, it seems very doubtful if it is really a component of the bone salts. More likely it is there accidentally, as an admixture from the body fluids. In bone ash preparations made by leaching the organic matrix with the alkaline glycerol reagent the KOH is undoubtedly an impurity. If we take Weiser's (19) analysis of bone ash obtained by incineration, we may assume that bone

might contain approximately 0.2 per cent of potassium as a constant component and that the considerably higher values obtained in our analyses must be attributed to small amounts of KOH from the reagent which were not completely washed out. Should we even allow that about 0.2 per cent is present originally in the bone mineral matter in the form of K_2CO_3 , this correction again would have no material effect upon the calculated composition. Furthermore, as far as the acid-base balance of the bone ash is concerned, this is not at all affected by either assumption.

Examining the data of the analyses recorded in Table II, giving the composition of vertebræ obtained from a variety of animals beginning with the cartilaginous fish (dogfish) and extending through the bony fishes, amphibians, reptiles, birds, and mammals, one is impressed by the remarkable similarity of the bone ash composition. The most striking difference which manifests itself in this series of comparative analyses is the relatively low $CaCO_3$ content of the vertebræ from marine fishes, with a $Ca_3(PO_4)_2:CaCO_3$ ratio which in round numbers is 12:1, whereas in all the other animals studied, owing to the considerably higher carbonate content, this ratio is much lower and is on the average 6.6:1. The low $CaCO_3$ content of the skeletal structure of marine fishes is probably due to the extremely low bicarbonate reserve of their body fluids. The residual calcium values (Ca other than $CaCO_3$) do not bear the relation to those of the residual P values (total P minus P in the form of $Mg_3(PO_4)_2$) as would be expected if the residual substance were nothing but $Ca_3(PO_4)_2$. In the latter case, the theoretical Ca:P ratio would be 1.935 whereas the ratios found invariably in the analyses of the salts of the vertebræ range from 1.99 to 2.12. In other words, a certain amount of calcium found in the analyses cannot be accounted for either as carbonate or phosphate. The calculated excess of Ca is recorded in the last column of Table II.

Turning now to Table III, we observe that the total basic equivalents of the ash exceed the total acid equivalents, as shown in Column 8. In general there are about 19 basic to 18 acid equivalents. Although neither Cl nor F was determined, both these radicles are found in such minute amounts in bone ash that their presence may be neglected without affecting the equivalent balance. Similarly the data obtained from titrating the bone

ash solutions corroborate the presence of such an excess of a basic component in the bone ash. The data recorded in Columns 9, 10, and 11 of Table III require a word of explanation. On dis-

TABLE II
Elementary Composition of Bone Ash Expressed in Per Cent

Material	Composition of bone ash					Calculated composition					
	Ca	Mg	K	P	CO	Mg ₂ (PO ₄) ₂	CaCO ₃	Ca ₃ (PO ₄) ₂	Ca(PO ₄) ₂ CaCO ₃	Residual Ca:P	Excess Ca
Dogfish	37.00	0.58	0.44	16.71	3.23	2.09	7.34	80.00	10.9	2.12	3.06
Goosefish	36.90	0.55	0.56	16.98	3.19	1.99	7.27	82.50	11.4	2.05	2.08
Mackerel	36.74	0.76	0.20	17.42	2.79	2.74	6.35	83.85	13.2	2.04	1.90
Squeteague	35.68	0.62	1.31	16.97	3.11	1.96	7.04	82.20	11.6	2.00	1.03
Frog (common)	36.09	0.72	0.44	16.45	4.67	2.59	10.62	79.15	7.5	2.01	1.20
Bullfrog	36.62	0.61	0.36	16.15	4.11	2.20	9.35	78.10	8.1	2.10	2.63
Amphiuma	36.86	0.51	0.76	15.96	5.34	1.84	12.13	77.60	6.4	2.06	2.01
Siren	36.96	0.57	1.05	16.00	5.73	2.06	13.02	77.55	6.0	2.05	1.75
Turtle	35.68	0.62	0.98	14.88	5.63	2.24	12.83	71.75	5.6	2.13	2.75
Hen	37.24	0.51		16.40	5.50	1.83	12.50	79.80	6.4	2.02	1.46
Rabbit	36.25	0.53	0.92	15.99	5.71	1.91	12.99	77.65	6.0	1.99	1.00
Cow	36.05	0.74	0.85	16.43	4.58	2.66	10.40	78.95	7.6	2.02	1.30
Dog	35.66	0.46	1.87	15.56	5.62	1.64	12.78	75.80	5.9	2.03	1.15
Guinea pig	35.54	0.75		15.78	5.03	2.71	11.44	75.65	6.6	2.04	1.66
Thyroid calculi	34.40	0.71		15.36	4.77	2.55	10.85	73.75	6.9	2.03	1.50
	34.60	0.63		15.80	4.51	2.27	10.22	76.30	7.5	2.00	0.91
Mixtures of tri-calcium phosphate (Eimer and Amend) and calcium carbonate	37.70			15.85	5.83		13.25	79.25	6.0	2.03	1.70
	36.44			15.64	4.83		11.00	78.20	7.2	2.05	1.89
	37.18			16.80	3.04		6.92	84.00	12.1	2.00	1.86

solving the bone ash in a measured volume of 0.1 N HCl the following reactions take place.

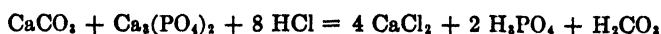


TABLE III
Balance of Acid and Basic Radicles of Bone Ash

Material	Equivalents per 1000 gm. ash										U. Li	ash
	Ca	Mg	K	PO ₄	CO ₂	Total	Difference (6-7)	Excess phosphate, n HCl	Excess HCl	Difference (11-8)		
	(1)	(2)	(3)	(4)	(5)	(6)	(8)		(11)	(12)		
Dogfish	8.50	0.48	0.11	6.16	1.47	9.09	7.63	1.46	3.29	2.36	0.93	0.53
Goosefish	8.45	0.46	0.14	6.15	1.45	9.05	7.60	1.45	2.87	2.36	0.51	0.94
Mackerel	8.37	0.63	0.05	6.83	1.27	9.05	8.10	0.95	2.90	2.54	0.36	0.59
Squeteague	7.84	0.52	0.34	6.40	1.42	8.70	7.82	0.88	3.13	2.70	0.43	0.45
Frog	8.05	0.60	0.11	5.89	2.12	8.76	8.01	0.75	3.49	2.83	0.66	0.09
Bullfrog	8.31	0.51	0.09	5.88	1.87	8.91	7.75	1.16	2.98	2.54	0.44	0.72
Amphiuma	8.43	0.42	0.19	5.57	2.45	9.04	8.02	1.02	3.27	3.02	0.25	0.77
Siren	8.48	0.47	0.27	5.47	2.61	9.22	8.08	1.14	3.44	3.20	0.24	0.90
Turtle	7.84	0.52	0.25	4.35	2.55	8.61	6.90	1.71	3.35	2.36	0.99	0.72
Hen	8.62	0.42	0.20	6.33	2.50	9.24	8.83	0.41	3.73	3.59	0.14	0.27
Rabbit	8.12	0.44	0.23	5.28	2.59	8.79	7.87	0.92	3.65	3.01	0.64	0.28
Cow	8.03	0.62	0.21	5.88	2.08	8.86	7.96	0.90	3.45	2.88	0.57	0.33
Dog	7.83	0.36	0.48	4.95	2.55	8.69	7.50	1.19	3.09	3.00	0.09	1.10
Guinea pig	7.77	0.63	0.20	5.78	2.29	8.60	8.07	0.53	3.09	3.01	0.08	0.45
Thyroid calculi	7.20	0.59	0.20	4.85	2.17	7.99	7.02	0.97	3.07	2.87	0.80	0.17
	7.30	0.63	0.20	5.56	2.05	8.13	7.61	0.52	3.06	2.63	0.43	0.09
Mixtures of tricalcium phosphate	8.85			5.36	2.64	8.85	8.00	0.85	3.78	2.88	0.90	0.05
(Eimer and Amend) and calcium carbonate	8.22			5.11	2.20	8.22	7.33	0.89	2.49	2.28	0.21	0.68
	8.59			6.70		8.59	8	0.51	3.30	2.52	0.78	0.27

In addition to these reaction products the mixture, of course, will contain the unused excess of 0.1 N HCl. In titrating this with 0.1 N NaOH to methyl orange 1 equivalent of the H_3PO_4 is determined together with the excess HCl. In Column 9 the titration results are recorded calculated in terms of N HCl reacting with 1000 gm. of the bone ash. Knowing from the analyses the total carbonate and phosphate content of the dissolved material, one can easily calculate the required quantity of HCl. Allowance is also made for the extra base in the form of KOH. The calculation can be best illustrated by an example.

250 mg. bone ash dissolved in.....	50 00 cc. 0.1 N HCl
0.1 N NaOH required for titrating back to methyl orange.	15.70 "
Used up by 250 mg. bone ash	34.30 1. 0.1 N HCl
" " " 1000 gm. " "	13.72 " N HCl
Acid used up to dissolve carbonates	2 50 "
	11 22 " N HCl
Acid used up to dissolve phosphates ($\frac{1}{2}$ equivalent) ..	10 88 "
	0 34 " N HCl
Acid used up to neutralize KOH.	0.20 "
Excess acid used up..	0.14 " N HCl

In this particular instance the analysis of the bone ash showed an excess of basic over acid radicles of 0.41 equivalent. In Column 10 of Table III the total correction for carbonates, phosphates, and KOH is recorded as a single value to save space.

It will be noted that the titrations indicate consistently the presence of a basic component in the bone ash, the acid used up in the titrations being invariably greater than demanded by the reaction. We have already seen that the analyses of the ash for Ca and P also show an excess of the former, if the Ca and P were components of a substance like $Ca_3(PO_4)_2$. To be sure, the excess basicity of the ash, as determined by titration, and the excess Ca as determined from the analysis, are not exactly equivalent to each other. Nevertheless, when one bears in mind that the inherent errors instead of compensating may be and frequently are cumulative, the coincidence of the results of direct analysis and of calculation from titration is most significant. Similar results were also obtained from the study of the calcified

masses from the thyroid gland as reported in Tables II and III. These facts point unavoidably to the conclusion that biological calcareous structures contain a basic calcium compound in addition to the other components, $\text{Ca}_3(\text{PO}_4)_2$ and CaCO_3 .

DISCUSSION

When the various view-points regarding the nature of the bone salts are surveyed, certain principal trends can be recognized. The oldest conception, first proposed by Hoppe (5) and later elaborated by Werner (20), assumes the existence in bone of a complex salt consisting of calcium carbonate and phosphate, $\text{Ca}[\{\text{Ca}(\text{PO}_4)_2\}_3]\text{CO}_3$, which places it in the apatite series of minerals. de Jong (7), Taylor and Sheard (18), and recently also Roseberry, Hastings, and Morse (14) by means of x-ray spectrum analysis of bone material brought forth evidence in favor of the above hypothesis of the apatite crystalline structure of bone salts, as was originally postulated by Hoppe. Taylor and Sheard even consider the crystal structure of the bone to be closely related to that of the apatite mineral podolite. Another view-point postulates that the bone salts may be represented as a combination of secondary CaHPO_4 and CaO , or a basic secondary phosphate (17). However, there is no evidence of the existence of CaHPO_4 in the bone ash. Taylor and Sheard and Roseberry, Hastings, and Morse pointed out that their studies fail to support such an assumption. Apart from such lack of support from physical studies on bone, the chemical composition of the bone ash cannot be brought into agreement with the CaHPO_4 hypothesis. Furthermore, if CaHPO_4 were present in bone it would upon incineration be converted to pyrophosphate and could not be determined until it had been changed back to orthophosphate by acid hydrolysis. This, however, we did not find to be the case with our bone ash material. On the contrary, when $(\text{NH}_4)_2\text{HPO}_4$ is precipitated slowly and with constant stirring with CaCl_2 , while maintaining a practically constant pH of 7.5 by the addition of sodium hydroxide, we obtained a substance which was proved to consist of $\text{Ca}_3(\text{PO}_4)_2$ and CaHPO_4 and which by analysis was found to contain about 14 parts by weight of $\text{Ca}_3(\text{PO}_4)_2$ and 1 part of CaHPO_4 , giving thus a molar ratio of 6.0:1.0.

Does the bone ash represent a complex calcium phosphate-

carbonate salt? In spite of various claims in favor of its existence based upon investigations by physical methods (7, 14, 18), the results of chemical analysis do not support such a view. In the first place, as we have found in the series of comparative analyses, the $\text{Ca}_3(\text{PO}_4)_2:\text{CaCO}_3$ ratio is radically different in marine fishes than in all other animals examined, the low calcium carbonate content of their bone ash bearing a very suggestive relationship to the extremely low bicarbonate reserve of the blood in those fishes. If bone ash were a definite crystalline compound of the carbonate and phosphate of calcium, its formation might be hastened or delayed by variations in concentration of the essential components dissolved in the blood serum, but would this affect the composition of the crystal? Furthermore, the phosphate:carbonate ratio does not correspond to the theoretically expected ratio of 9.3 and is variable, being apparently influenced by different physiological conditions (it is markedly lowered by rickets (6) and is altered by the state of nutrition, by age, and by phosphorus deficiency in the diet (10, 11)).

Our analytical studies bring out the significant fact that bone ash contains more calcium than can be accounted for on the basis of carbonate and phosphate and, furthermore, that this excess of calcium must be in the nature of a basic compound which uses up acid in the titration. Our method of preparation of the bone ash precludes the possibility that this excess might be derived from Ca collagenate of the bone matrix. In this respect the bone ash composition is similar to that of a tricalcium phosphate (Eimer and Amend, Tested Purity grade) which on analysis also showed an excess of 1.64 equivalents of Ca and which by our titration procedure was found to be a basic Ca. This purchased tricalcium phosphate was, therefore, quite different from the material which we ourselves prepared in that it contained $\text{Ca}(\text{OH})_2$ instead of CaHPO_4 although in the same molar ratio.

Turning once more to the data reported in Table II and guided by the evidence of the presence of a basic calcium compound in the material, we may now attempt to determine the composition of the bone ash on the basis of this chemical information. For this purpose the average value for separate groups rather than for each separate sample will be considered. From the results of the analyses of the bone ash of marine fishes the average composition

corresponds to 82.15 per cent $\text{Ca}_3(\text{PO}_4)_2$, 7.00 per cent CaCO_3 , and 3.74 per cent $\text{Ca}(\text{OH})_2$. In terms of mols these values are 82.15:310, 7.00:100, and 3.74:74, thus giving a molar ratio of 6.00:1.57:1.13 which is most nearly satisfied by the composition $\text{Ca}[\{\text{Ca}_3(\text{PO}_4)_2\}_6](\text{OH})_2$. Similarly, from all the other bone ash analyses an average composition is obtained of 77.20 per cent $\text{Ca}_3(\text{PO}_4)_2$, 11.81 per cent CaCO_3 , and 3.13 per cent $\text{Ca}(\text{OH})_2$. This corresponds to a molar ratio of 6.00:2.82:1.02, which satisfies the above formula even more perfectly. For the artificial mixtures of CaCO_3 and $\text{Ca}_3(\text{PO}_4)_2$ (Eimer and Amend) the composition corresponds to a molar ratio of 6.00:2.86:1.06, for the first two, and of 6.00:1.53:1.04 for the third mixture, for the $\text{Ca}_3(\text{PO}_4)_2$, CaCO_3 , and $\text{Ca}(\text{OH})_2$ respectively. In other words, the Eimer and Amend tricalcium phosphate product, like the bone ash material, seems to be correctly described by the formula $\text{Ca}[\{\text{Ca}_3(\text{PO}_4)_2\}_6](\text{OH})_2$. It may be recalled that the tricalcium phosphate which we ourselves prepared contained CaHPO_4 , the molar ratios being practically 6.00:1.00, so that it likewise might be described as the following compound, $\text{Ca}[\{\text{Ca}_3(\text{PO}_4)_2\}_6]\text{HPO}_4$, in which CaHPO_4 takes the place of the $\text{Ca}(\text{OH})_2$.

It is not easy to apply this mode of calculation to bone ash analyses reported by other authors owing to the fact that essential data are generally lacking as, for instance, the CO_2 content, where the analyses have been made on incinerated bone. We have taken, however, one of Shear and Kramer's analyses on whole bone material ((15) p. 114) and, introducing a correction for magnesium, we find that the composition of that particular sample of bone was 51.00 per cent $\text{Ca}_3(\text{PO}_4)_2$, 9.21 per cent CaCO_3 , and 2.09 per cent $\text{Ca}(\text{OH})_2$. The latter was calculated from the calcium not accounted for as carbonate and phosphate. This relative composition corresponds to a molar ratio of 6.00:3.30:1.00, thus corroborating in a general way what we have found in our analyses of bone ash.

SUMMARY AND CONCLUSION

A comparative investigation of the vertebræ has shown that the principal difference between the chemical composition of the bone ash in marine fishes and in a variety of higher vertebrates is in the proportion of CaCO_3 , which is only about one-half as large as in the latter.

Evidence is adduced to show that the bone ash does not contain CaHPO_4 . However, the bone salts do not consist merely of CaCO_3 and $\text{Ca}_3(\text{PO}_4)_2$ but very probably also of $\text{Ca}(\text{OH})_2$. Since analyses do not reveal any definite and simple molar relationship between the CaCO_3 and $\text{Ca}_3(\text{PO}_4)_2$ the hypothesis that a crystalline complex salt $\text{Ca}[\{\text{Ca}_3(\text{PO}_4)_2\}_3]\text{CO}_3$ is the basis of bone structure seems improbable. On the contrary, a definite and simple molar relation between the $\text{Ca}_3(\text{PO}_4)_2$ and $\text{Ca}(\text{OH})_2$ of 6.00:1.00 does point strongly to the possibility that the principal component of the bone ash is a complex salt of the nature $\text{Ca}[\{\text{Ca}_3(\text{PO}_4)_2\}_3](\text{OH})_2$.

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DIFFUSIBILITY OF THE PROTEINS OF NORMAL AND PATHOLOGICAL PLASMA

By OLIVER HENRY GAEBLER

(From the Department of Laboratories, Henry Ford Hospital, Detroit)

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The excretion of protein during nephritis is commonly ascribed to changes which have taken place in the kidney, rather than to changes in the proteins or other constituents of plasma. Clausen has, however, obtained a substance from urine of cases of parenchymatous nephritis which is said to increase the permeability of collodion membranes to protein (1). He believes that this substance, which lowers the surface tension of blood, may be a factor in the production of the marked albuminuria seen in such cases (2). In studies of other types of nephritis, Welker, Andrews, and Thomas (3) record the observation that when nephritic blood is dialyzed, protein material passes into the dialysate, while in the case of normal blood this does not occur. They do not, however, suggest that this difference is due to the action of some substance on the membranes, but possibly to increased dispersion of the proteins resulting from combination with toxic substances. Norris (4) found no evidence that substances which lower the surface tension increase the permeability of membranes.

It thus appeared to be of interest to carry out a series of simple dialysis experiments with blood plasma from normal subjects and from patients with and without albuminuria. The necessary conditions for comparisons are that the membranes used should be uniform, and that the amount of protein which diffuses in a given time should be directly proportional to the concentration dialyzed. Since dialysis experiments with membranes of high permeability and with substances of a protein character have not been very common, experiments were carried out to determine the extent to which the above conditions could be met.

Methods

The dialysis cells used were constructed from pieces of builders' cork which were 3 inches square and of uniform half inch thickness. Two pieces served as ends. From three others, 1 × 2 inch pieces were sawed out (Fig. 1). All the pieces were soaked in melted paraffin and the ends were fastened on with this substance. The surfaces coming in contact were covered with vaseline, and the three parts placed together with flat collodion membranes between, thus forming a three-chambered cell. 10 cc. of plasma or protein solution were placed in the central compartment, and 10 cc. of 0.8 per cent sodium chloride solution or water in the lateral compartments. In each experiment the protein solution was thus tested with two membranes simultaneously. The dimensions of

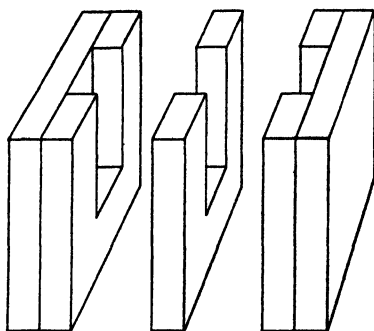


FIG. 1. Dialysis cell

the chamber and amounts of solutions are such that an area somewhat more than an inch square, and in the center of the membranes, is actually exposed to the liquids.

The flat collodion membranes were prepared by the Walpole method as developed by Nelson and Morgan (5), with minor modifications. Du Pont's parlodion, after removal from the water in which it is shipped, was dried in air for 4 to 5 days. Weight loss after this period is slight. 8 gm. of the dry parlodion were dissolved in a mixture of 300 gm. of absolute alcohol and 100 gm. of purified anhydrous ether. 5 cc. of the resulting solution were delivered from a fast flowing pipette upon 4 inch circular pieces of plate glass, previously weighed, and perfectly leveled. This

amount of solution spreads very nearly to the edge of the plate. A small tag bearing the number of the membrane and at the same time identifying the side which was uppermost during evaporation was immersed in the edge of the film. After 5 minutes the plate and membrane were transferred to the balance pan, and allowed to remain until the membrane weighed 2.1 gm. Immersion in distilled water or alcohol solutions followed immediately.

The plates were not fogged, as in the experiments of Nelson and Morgan, and contrary to the experience of these workers the membranes came off the plates spontaneously or with negligible difficulty except in rare instances. The usual cleaning with sulfuric acid-dichromate mixture did not suffice to produce this result. The plates were placed in concentrated sulfuric acid-dichromate mixture, heated to about 140° , then allowed to cool and remain in the mixture overnight. Thereafter they were rinsed with distilled water, dried at 110° for a very short time, and cooled in air. Settling of visible dust on the plates must be avoided.

No device for mixing the solutions during dialysis was used. The dialysis cell was simply covered with a watch-glass and placed upon the laboratory desk. The duration of dialysis was in all cases 4 hours, after which period the solutions were removed with pipettes, measured, and analyzed.

The collodion solutions were prepared in such amounts that they were used up in about 1 month. Dry weights of membranes from the first and last portions did not differ; hence evaporation during the frequent removal of samples was negligible. Tests with a given protein preparation showed no noteworthy changes in permeability of the membranes due to age of the collodion solution, during the time stated.

In the course of preliminary experiments with over 100 membranes many of the observations of Nelson and Morgan were repeated with entirely similar results. Thus the final grade, or gm. of water per gm. of parlodion in the membranes which had been passed through alcohol solutions previous to immersion in water, averaged 22.2 as compared with 22.8 in the experiments cited. Similarly the ratio of the grade before immersion in alcohol to that after subsequent removal from water averaged 1.24 as compared with 1.21, and the dry weight 74 mg., as compared

with 76 mg.¹ The membranes were therefore entirely comparable, but the experiments to be reported involve conditions and substances quite different from those in the work of Nelson and Morgan.

Unequal Permeability of Flat Collodion Membranes in Opposite Directions

In the first experiments the membranes were always evaporated to 2.1 gm., then plunged directly into distilled water and transferred to another beaker of this when they came off the plates. After several hours they were used. Such membranes show an

TABLE I

Showing That Membranes Immersed Directly in Water Lack Uniformity and Are Unequally Permeable in Opposite Directions

Experiment No	Top of membrane toward protein		Bottom of membrane toward protein	
	Membrane No.	Concentration of protein outside	Membrane No	Concentration of protein outside
		<i>per cent</i>		<i>per cent</i>
1	42	0.042	45	0.099
2	44	0.057	46	0.094
3	49	0.070	48	0.137
4	57	0.032	53	0.127
5	56	0.033	55	0.114
6	54	0.042	58	0.130
Average...		0.046		0.117

interesting asymmetry. If the side of the membrane which was uppermost during evaporation (designated as the top) is placed toward the protein solution, much less protein passes through than if the side which was toward the plate (bottom) is placed toward the protein.

Typical results are shown in Table I. In these experiments water solutions of Merck's "egg albumin, soluble" were used. The solutions contained 6.2 gm. of protein per 100 cc. ($N \times 6.25$). One of the two membranes used in each experiment was placed with the top, the other with the bottom toward the protein solu-

¹ The data in the lower half of Table V in the reference cited are taken as a basis of comparison.

tion. Dialysis against water was carried out for 4 hours. The protein concentration in the lateral chambers was then determined by precipitating a portion with sodium tungstate and sulfuric acid and applying the colorimetric procedure of Wu and Ling (6) to the precipitate. The tyrosine equivalent of the egg albumin preparation was found to be 19.8. The greater permeability of the membranes in the one direction is shown in every experiment in Table I. Apart from showing this gross difference, the membranes are, however, too irregular for simple dialysis experiments of this type, with substances that do not diffuse easily.

TABLE II
Showing the Uniformity of Alcohol-Treated Membranes

Experiment No.	Top of membrane toward protein		Bottom of membrane toward protein	
	Membrane No.	Concentration of protein outside	Membrane No.	Concentration of protein outside
		<i>per cent</i>		<i>per cent</i>
1	84	0.163	83	0.154
2	88	0.166	87	0.158
3	105	0.176	104	0.150
4	103	0.163	106	0.168
5	107	0.158	108	0.168
6	119	0.164	118	0.166
7	121	0.168	120	0.162
8	123	0.166	122	0.170
Average.....		0.166		0.162

The next experiments were carried out with another type of membrane described by Nelson and Morgan, in which the residual solvent is gradually, instead of suddenly replaced by water. The membranes were prepared exactly as before, but after evaporating to 2.1 gm., were placed successively, for 5 minute periods, in alcohol solutions containing by volume 95, 90, 80, 70, 60, 50, 40, and 30 per cent of alcohol, then for 10 minute periods in 20 and 10 per cent solutions, and finally in distilled water. The membranes came off the plates spontaneously in the distilled water and after a short time were transferred to a large beaker of distilled water, in which they remained overnight before being used. Dialysis experiments with the same egg albumin preparation were

again carried out, and the results were now quite different (Table II). The average transmission of the albumin is essentially the same whether the top or bottom of the membrane faces the protein solution. The membranes are also much more uniform, varying only ± 8.5 per cent from the average permeability. Repetition of the experiments in Table II with a second set of sixteen membranes yielded averages of 0.162 and 0.163 per cent, which correspond closely with those recorded.

When membranes of the above type were used with plasma, it was surprising to find that unequal permeability in opposite directions reappeared. It was, however, much less marked than in the case of membranes not immersed in alcohol solutions before immersion in water. Thus in twenty-eight experiments with plasma and serum, requiring 56 membranes, the unequal permeability in opposite directions was observed without exception, an average of 1.19 times as much protein being transmitted in the bottom to top direction as in the reverse. The variation of this ratio did not equal twice the previously observed variation of individual membranes, hence it was concluded that the membranes were as uniform in their behavior toward plasma proteins as toward commercial egg albumin.

In each of the following experiments with plasma, one of the two membranes used was placed with the top, the other with the bottom toward the plasma, and the average concentration of protein in the two lateral chambers after 4 hours was taken as "protein outside" in calculations. The technique was altered in that dialysis was carried out against 0.8 per cent sodium chloride solution, and that the protein determinations were carried out by digesting the tungstate precipitate and determining the ammonia by distillation and titration in a manner similar to that described by Howe (7).

Results with Diluted and Undiluted Plasma

Whether the amount of protein diffusing is directly proportional to the concentration dialyzed was tested as follows: A plasma sample was divided into two portions, one of which was dialyzed without further treatment; the other was dialyzed after dilution with an equal volume of 0.8 per cent sodium chloride solution. Eight different samples of plasma were tested in this way. The

results are given in Table III. Dividing the concentration of protein outside by the concentration inside of the membranes at the end of 4 hours yields practically the same average coefficient whether the plasma is diluted or undiluted. Since two membranes are used in each experiment, the averages of four figures represent the averages of eight membranes, which according to the preceding findings exclude individual variations.

Comparison of Normal and Pathological Plasma

In Table IV are shown the results of dialyzing series of plasma samples from normal individuals, from patients with little or no

TABLE III
Results with Diluted and Undiluted Plasma

Experiment No.	Concentration of protein outside Concentration of protein inside		Source of plasma
	Undiluted plasma	Diluted plasma	
1	0.0366	0.0408	Dog
2	0.0433	0.0372	"
3	0.0438	0.0462	"
4	0.0473	0.0405	"
Average.	0.0427	0.0412	
5	0.0413	0.0363	Human
6	0.0369	0.0338	"
7	0.0467	0.0455	"
8	0.0321	0.0328	"
Average.	0.0392	0.0371	

proteinuria or other evidence of renal disease, and from patients with marked proteinuria. The diffusibility of the plasma proteins is obtained as before by dividing the concentration of protein outside of the membranes by the concentration inside at the end of 4 hours, and is shown in the third column of Table IV. It is apparent that both the range and average of this coefficient are the same for plasma samples from the third group of subjects, which had marked proteinuria, as for the second group, in which proteinuria was negligible or absent. The cases of chronic nephritis were of quite different types. The urine samples of all con-

tained albumin and casts, and that of subject Ha contained large numbers of red blood cells. Subject Co belonged to the arterio-

TABLE IV
Comparison of Normal and Pathological Plasma Samples

Subject	Condition	Concentration of protein outside	Urine protein	Plasma proteins		
		Concentration of protein inside		Fibrinogen	Globulin	Albumin
			mg. per 100 cc	per cent	per cent	per cent
Ga	Normal	0.0430	0			
"	"	0.0467	0	0.22	1.84	4.60
Hi	"	0.0321	0	0.44	2.31	4.60
Zi	"	0.0468	0	0.28	1.98	5.00
Sc	"	0.0454	0	0.25	1.74	4.43
Average...		0.0428				
Mu	Lues	0.0363	7			
To	"	0.0291	5			
Sp	Convalescent pneumonia	0.0370	0			
Ad	Traumatic neurosis	0.0348	0	0.40	2.39	4.15
Bu	Postoperative gastroenterostomy	0.0353	0	0.36	2.67	3.63
Average...		0.0345				
Pa	Chronic nephritis	0.0356	75			
Ha	" "	0.0329	750			
Co	" "	0.0386	30			
Kr	" "	0.0328	980	0.40	2.99	3.05
Gan	" "	0.0305	500	0.48	1.76	2.72
Average ..		0.0341				
Ku	Nephrosis	0.0055	1000	0.69	2.53	1.48
"	"	0.0081		0.88	2.56	1.08
Ne	"	0.0093		0.51	1.54	1.55
"	"	0.0216	400	0.38	1.92	1.82
Average.....		0.0111				

sclerosis-hypertension group, and showed no nitrogen retention, while subject Kr showed marked nitrogen retention. Subject Gan

was similar to the cases of nephrosis—edema was slight, the albumin concentration in the urine subsequently approached 2 per cent, nitrogen retention was not marked (43 mg. per 100 cc. of blood), and plasma albumin was lowered, but cholesterol was not elevated. The patient was, however, in much better condition than the others, being in the hospital only for very short periods and continuing his regular work without apparent difficulty between admissions.

The plasma samples from normal subjects, all of whom were laboratory workers on duty at the time when the blood was drawn, show a considerably greater diffusibility of the proteins than any of the samples from patients.

Contrasting sharply with all of the other results are those obtained on four samples of plasma from two cases of so called nephrosis. These are shown at the foot of Table IV. Here the diffusibility, far from being increased, is markedly reduced. The two cases, one an adult and the other a child, presented most of the typical findings of the condition indicated—marked albuminuria and edema, little nitrogen retention, high blood cholesterol values, as well as the greatly lowered albumin and increased fibrinogen values shown in Table IV. The values for cholesterol in the whole blood were 0.213 and 0.348 per cent, the non-protein nitrogen values 56 mg. and 40 mg. per 100 cc., for subjects Ku and Ne respectively. The concentration of protein in the urine was often much greater than in the specimens accompanying the blood samples. The marked increase in diffusibility in the second sample from subject Ne followed a sudden disappearance of the edema, and general improvement, under treatment. A sufficient number of cases of this type has not as yet been studied to determine whether the observed changes in the diffusibility are referable to changes in the proteins or to the presence of other substances in the plasma which are taken up by the membranes. The fibrinogen values recorded were obtained by the method of Wu and Ling, and the globulin and albumin values by the method of Howe.

DISCUSSION

An unequal permeability of artificial membranes in opposite directions has been previously observed by Hamburger (8), who emphasized the point that the phenomenon is not confined to liv-

ing membranes. Wertheimer (9) carried out dialyses with bags prepared from the skin of frog legs, and compared the results obtained with "normal" and "reversed" membranes. He uses the term "irreciprocal permeability" to designate the difference in the two cases. In the present studies it is apparent that the membranes are least penetrable from the side which was exposed to evaporation and which came in contact with water first during preparation. Whether the phenomenon is observed, depends upon a number of factors—the substance tested, the treatment of the membrane, and possibly the duration of dialysis. The same type of membrane (alcohol-treated) which did not show the phenomenon with an albumin preparation did show it with plasma and serum. From this it seems probable that the difference in permeability is more readily observed with the larger globulin molecules. With the same albumin preparation, alcohol-treated membranes did not show the phenomenon, while those which were immersed directly in water did show it. Apparently the gradual replacement of the residual solvent in the membrane by immersion in graded alcohols minimizes this effect of water immersion as it minimizes the change in grade resulting from water immersion (5). The effect of duration of dialysis was not studied, but it should be borne in mind that after 4 hours the protein concentration outside is only 3 to 4 per cent of that inside, and that one is not dealing with a condition of equilibrium.

In connection with the observations on plasma it is shown in Table III that the factor of dilution of any given plasma can be largely excluded by the method of calculation adopted. It is, however, well known that a fall in plasma protein is not a simple dilution. In the last group of subjects in Table IV the fibrinogen and globulin values are higher, and the albumin values far lower than in the normal subjects, and for such changes the method does not correct. But in investigating a possible relationship to proteinuria, one should perhaps disregard the concentration of plasma protein, and simply answer the question: How much protein passes from uniform volumes of plasma through uniform areas of membrane in a given time? When this is done the explanation of proteinuria on this basis becomes even less evident. For the four groups in Table IV the averages of the concentration of "protein outside" after 4 hours were 0.214, 0.177, 0.149, and

0.039 per cent, respectively. The inverse relationship between the amount of protein which diffuses and the gravity of the proteinuria becomes more striking, simply because the samples of plasma from cases showing marked proteinuria contain less protein.

SUMMARY

1. Flat collodion membranes of high permeability show a different permeability for protein in opposite directions.

2. Alcohol-treated membranes prepared according to the technique of Nelson and Morgan were found very suitable for dialysis experiments with protein.

3. Simple dialysis experiments on plasma with such membranes yielded no evidence that proteinuria is due to an increase in the diffusibility of plasma proteins or to the presence of substances increasing the permeability of artificial membranes.

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STUDIES OF THE PEPTIDES OF TRIVALENT AMINO ACIDS

I. TITRATION CONSTANTS OF HISTIDYL-HISTIDINE AND OF ASPARTYL-ASPARTIC ACID

By JESSE P. GREENSTEIN*

(From the Department of Physical Chemistry in the Laboratories of Physiology, Harvard Medical School, Boston)

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INTRODUCTION

While numerous investigators (1-4) have studied the dissociation of amino acids and a few simple peptides, little or no data are available on the more complex peptides involving those trivalent amino acids such as histidine, arginine, lysine, and the dicarboxylic acids, which furnish the free groups to proteins. Clearly, any attempt to correlate the acid- and base-combining capacity of a protein and the strength of the apparent dissociation constants with the number of free valencies, cannot be satisfied with data on the simple ampholytes alone. It would be advantageous to supplement such information by a study of the manner in which several of such free groupings behave when congregated in a synthetic model, which by the number of its extra valencies, bears the closer analogy to the protein.

Attempts have been made to analyze protein titration curves in terms of dissociation constants ever since the early investigations of D'Agostino and Quagliariello (5). The titration curves of many proteins were partially discussed in terms of dissociation constants by Cohn (6, 7) and the significance of these constants elaborated in terms of the *Zwitter Ionen* hypothesis by Simms (8, 9). The fact that in most of the curves reported, the extreme acid and alkaline constants coincided, approximately, within the range of the corresponding acid and basic constants of amino

* National Research Council Fellow in Biochemistry.

acids, led inevitably to attempts to analyze the titration curve in terms of the dissociation of simple ampholytes. Simms (8) related the apparent dissociation constants of several proteins to the ionization furnished by the free groups of the trivalent amino acids, *i.e.* those groups not involved in polypeptide linkage. In order to account for a titration index at pH 4.6, he presented the hypothesis that arginine existed "preformed" in the molecule, the amino acid appearing only secondarily as a result of hydrolysis. While Simms may be correct in his view that the titration value of 4.6 in gelatin and in egg albumin is due to a basic and unsaturated compound, it is probable that such value may be due to the position of the basic molecules held in peptide linkage.¹ That these and similar constants occurring in ranges not possessed by amino acids, may be due to the influence of neighboring groups upon the groups of amino acids held in peptide combination, had been advanced earlier by Cohn (7).

While electrostatic forces may account in part for the shifting of the values of characteristic groups in amino acids to new ranges demonstrated by the protein, the influence of the peptide linkage plays an important rôle. Cohn (7) pointed out that in the titration curves of the proteins studied, the extreme acid constant varied only from 2.8 to 3.7, the extreme basic constant from 10.2 to 11.1. These values are at the same time weaker than those of the carboxyl group of the aliphatic amino acids (about 2) and the guanidine group of arginine (about 13). Such a decrease in the strength of the acid and basic groups is comparable with that shown by corresponding groups in the transition of amino acids to peptides, and points to a diminution of the energy of ionization acting through longer chains of atoms (4). Furthermore, the change in the strength of a given group in an amino acid is greatest when the acid is combined with another to form a peptide; addition of another amino acid to form a tripeptide alters its strength very much less, while further lengthening of the chain produces only extremely small changes in its value (10). Thus it seems likely that the titration values noted in the protein may

¹ The groups of certain proteins containing a large number of basic amino acids dissociate at more acid reactions than can be accounted for on the basis of their composition and the dissociation constants of the free amino acids.

be related to the ionizable groups in the amino acids, not when the latter are in the free state, but only when combined in peptide linkage.

Accordingly it was felt worth while to prepare and study the peptides of histidine and of aspartic acid which would in the former case yield three free basic and one free carboxyl groups, in the latter one free amino and three free acid groups. These of all synthetic peptides should most closely resemble the protein molecule in the matter of crowded free charges if not molecular size. Further studies in peptides of the more basic amino acids are now in progress.

For purposes of comparison the titration constants of histidine dihydrochloride and of aspartic acid were redetermined. The constants calculated are comparable with those reported by previous investigators.

EXPERIMENTAL

Preparation of Materials—The histidine dihydrochloride used for the titration reported herein and for the synthesis of histidyl-histidine, was prepared from hemoglobin by the method of Vickery and Leavenworth (11). Horse hemoglobin was crystallized and purified by the method of Ferry and Green (12), and after HCl hydrolysis the histidine was separated as the silver salt at pH 7.2. A yield of crude histidine of a little over 7 per cent from 200 gm. of dried hemoglobin was obtained. The crude product was purified as the dihydrochloride salt and gave 6.11 per cent of amino nitrogen, theory 6.14 per cent, m. p. 235° uncorrected.

Histidyl-histidine was prepared according to the method of Fischer and Suzuki (13) as modified by Pauly (14). The methyl ester of histidine, instead of being heated in a closed tube to yield the anhydride, was heated at 100° *in vacuo*. The slightly colored mass obtained was crystallized once from hot water and gave 0.78 per cent amino nitrogen. The mother liquor appeared to contain a considerable amount of crude histidine. A second crystallization from a large amount of solvent yielded a pure product, which after drying *in vacuo* at 80°, gave no amino nitrogen whatever, m.p. 310° uncorrected. An amount of 0.2740 gm. of pure histidine anhydride was suspended in 15 cc. of N NaOH, and the flask shaken for 24 hours. Complete solution apparently took

place after the 1st hour. At the end of the 24 hour period of shaking, samples were removed at intervals over another 24 hour period of standing. The samples were diluted with water and appropriate aliquots employed for amino nitrogen analysis. Blanks of the same alkalinity were employed. The remarkable quantitative conversion of the anhydride to the peptide, together with the latter's stability in the presence of a large amount of normal alkali, was shown by the data. After the first 24 hour period of shaking, the amino nitrogen was 4.87 per cent, after the end of the 2nd day it was 4.82 per cent; theory for histidyl-histidine, 4.80 per cent. The stoichiometric character of the titration curve later confirmed these analytical data. Fischer and Suzuki had succeeded in isolating the peptide from the neutralized solution as the dipicrate but this isolation was considered unnecessary for the present purpose.

The aspartic acid used in the titration reported was an Eastman product and after purification gave 10.52 per cent of amino nitrogen, theory 10.52 per cent.

Aspartyl-aspartic acid was prepared from a good commercial grade of asparagin by the method of Fischer and Koenigs (15). The yield of 2,5-diketopiperazine-3,6-diacetic dimethyl ester was quite high and the material was purified by several recrystallizations from slightly acidulated water. The saponification of the diester was followed by the micro-Kjeldahl method of Koch and McMeekin (16), the total nitrogen figures for the diacid yielding 12.0 per cent; theory 12.2 per cent; theory for the dimethyl ester, 10.8 per cent. The anhydride was previously dried at 80° *in vacuo*, and gave no amino nitrogen whatever; m.p. 270° uncorrected. A sample of 0.1150 gm. of aspartic anhydride was suspended in 7.5 cc. of N NaOH and shaken for 24 hours. At the end of this period amino nitrogen determinations were taken as before, yielding 5.61 per cent, and after another 24 hour period in contact with the alkali, again 5.61 per cent; theory for aspartyl-aspartic acid, 5.64 per cent. Fischer and Koenigs had isolated this peptide in the pure state but it was felt undesirable to go to this length inasmuch as the repeated treatment of the peptide with absolute alcohol, as the authors pointed out, led to a partial esterification of the compound. Although Fischer and Koenigs' analytical figures for the isolated peptide checked with those

calculated for aspartyl-aspartic acid, and although the method of preparation led them to the belief that this was the compound sought, they apparently remained somewhat uncertain regarding their product. It may be mentioned that neither the amino nitrogen technique nor the formol titration had been developed at that time. At the close of their article Fischer and Koenigs remarked, "die Struktur. . . ist abgeleitet aus der Formel des Piperazinkörpers. Wir halten es aber für sehr wünschenswert, dass sie durch eine gründliche Untersuchung der Säure geprüft wird." Together with the present total and amino nitrogen figures and the electrometric titration, which revealed the presence of exactly four free groups in the molecule, the structure of this compound would appear to have been proved. There no longer appears to be any doubt, therefore, regarding the adequacy of this elegant synthesis of Fischer's.

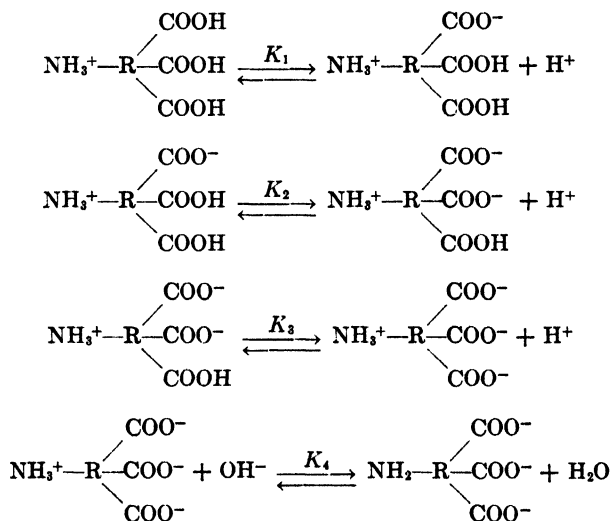
Procedure—The dissociation of these compounds was studied by means of the hydrogen electrode. The electrode was of the bubbling type, the platinum very thinly coated. A 0.1 M KCl electrode similar to the model of Lewis, Brighton, and Sebastian was employed, and was standardized before each titration with 0.1 M HCl. Scatchard's value for $p_{\gamma_{H^+}}$ of 0.076 (17) for 0.10 M HCl was adopted. The calomel cell barely dipped into a saturated KCl reservoir which in turn was connected to the hydrogen half-cell by means of a saturated KCl agar bridge. A 1 cc. burette graduated in hundredths was inserted in the stopper of the hydrogen electrode vessel. A water bath maintained the temperature at 25°.

In the histidine dihydrochloride study, 5 cc. of a 0.1 M solution of the salt were titrated with normal NaOH. Aspartic acid was made up to 0.02 M and 5 cc. titrated respectively with 0.5 M HCl and 0.5 M NaOH.

As described previously, the peptides of the amino acids were not isolated but were studied after the splitting with alkali of the piperazine ring. Exactly 0.1370 gm. of histidine anhydride was suspended in 7.5 cc. of N NaOH in a 25 cc. volumetric flask and shaken gently for 24 hours at room temperature. At the end of this period 7.5 cc. of N HCl were added and then distilled water until the volume was 25 cc. This procedure yielded a 0.02 M solution of the peptide in the presence of 0.3 M NaCl. 5 cc.

samples were titrated with 0.5 M HCl or 0.5 M NaOH. The same procedure was employed with aspartic anhydride, with that weight of the diketopiperazine, namely, 0.1150 gm., which would give a 0.02 M solution of the peptide when the volume had been brought to 25 cc.

Method of Calculation—If we assume that the ampholyte dissociates in several stages with the splitting off of hydrogen ion, a quadrivalent ampholyte such as aspartyl-aspartic acid may be represented as follows:



In the first dissociation, the fraction of ampholyte salt found will be

$$\frac{\text{NH}_3^+ - \text{R} \begin{array}{l} \text{COO}^- \\ \text{COOH} \\ \text{COOH} \end{array}}{C} = \alpha_1$$

where C is the total concentration of ampholyte. In acid solutions, $\alpha_1 = \frac{A - \text{H}^+}{C}$, where A is the mols of acid added and H^+ is the hydrogen ion activity as measured by the reversible electrode (4). To express the relation entirely in terms of concentration,

the hydrogen ion activity is divided by the activity coefficient of the hydrogen ion at the same total ionic strength.² Likewise for the fourth dissociation,

$$\alpha_4 = \frac{\text{NH}_2-\text{R}-\begin{array}{c} \text{COO}^- \\ \text{COO}^- \\ \text{COO}^- \end{array}}{C} = \frac{B - \text{OH}^-}{C}$$

where B is the mols of base added and where OH^- , the activity of hydroxyl ions measured, is converted into concentration by dividing by the appropriate activity coefficient.²

The dissociation of each group is measured by α , when the former dissociates like a monovalent acid or base. The solution of the ampholyte is assumed to consist of a mixture of monovalent acids and bases in equivalent proportions, each ionizing independently of the others. The apparent titration constant of each group may be expressed therefore in terms of the mass action law:

$$G' = H \frac{\alpha}{1 - \alpha} \text{ or } \text{p}G' = \text{pH} - \log \frac{\alpha}{1 - \alpha}$$

² For aspartic acid $\text{p}\gamma_{\text{H}^+}$ was calculated from the results of Scatchard's study (17) of pure HCl solutions. $\text{p}\gamma_{\text{OH}^-}$ was taken from a smooth curve drawn through Lewis and Randall's data (18) for pure NaOH solutions.

For histidine, histidyl-histidine, and aspartyl-aspartic acid, which were titrated in the presence of NaCl, $\text{p}\gamma_{\text{H}^+}$ and $\text{p}\gamma_{\text{OH}^-}$ were determined experimentally. Salt solutions of concentration identical with those containing ampholyte were titrated with the appropriate concentrations of acid or alkali. The negative logarithms of the activity coefficients were obtained from the respective equations

$$\begin{aligned} \text{p}\gamma_{\text{H}^+} &= \text{pH} - \text{pA} \\ \text{p}\gamma_{\text{OH}^-} &= \text{p}K_w - \text{pH} - \text{pB} \end{aligned}$$

where A is the total acid, B the total base concentration, and where $\text{p}K_w$, the dissociation constant of water at 25° was taken as 13.998. The values of $\text{p}\gamma$ were plotted against the square root of the ionic strength and smooth curves drawn through the points. It seemed evident that while neutral salt in moderate concentration increases the activity coefficient of the hydrogen ion, that of the hydroxyl ion is diminished.

The activity coefficient was assumed to be independent of the presence of the ampholyte. While this is not strictly accurate it must suffice for the present. The problem of the contribution of the ampholyte to the ionic strength awaits a comprehensive study of the ampholyte in cells without liquid junction.

When the ampholyte is considered as a whole, with the splitting off of hydrogen ions without reference to any particular group or groups, the dissociation equilibrium may be represented in terms of the classical dissociation constant, K' . If the titration constants of the ampholyte are sufficiently far apart in value, the two classes of constants are identical. When, however, two or

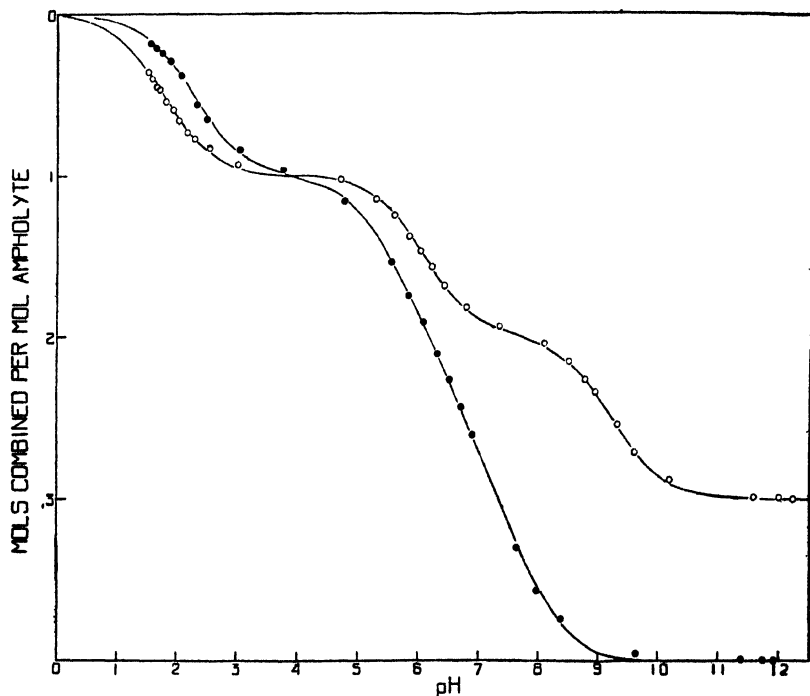


FIG. 1. Curves constructed on the basis of the constants: histidine, $pG_1' = 1.77$, $pG_2' = 6.10$, $pG_3' = 9.18$; histidyl-histidine, $pG_1' = 2.25$, $pG_2' = 5.60$, $pG_3' = 6.80$, $pG_4' = 7.80$. ● indicates histidyl-histidine; ○, histidine.

more steps in the ionization occur simultaneously, the constants are no longer identical. The pragmatic approach is then to treat that segment of the titration curve over which the overlapping occurs as the resultant of the simultaneous dissociation of two or more monovalent acids or bases. The constants of each of these acids or bases will be the titration constants G' . The total

amount of acid or base bound at any one pH value by the ampholyte per mol of ampholyte then may be expressed as:

$$x = \alpha_1 + \alpha_2 + \dots \alpha_n = \sum \alpha$$

The relation between the titration constants and the classical dissociation constants was first clearly demonstrated by Simms

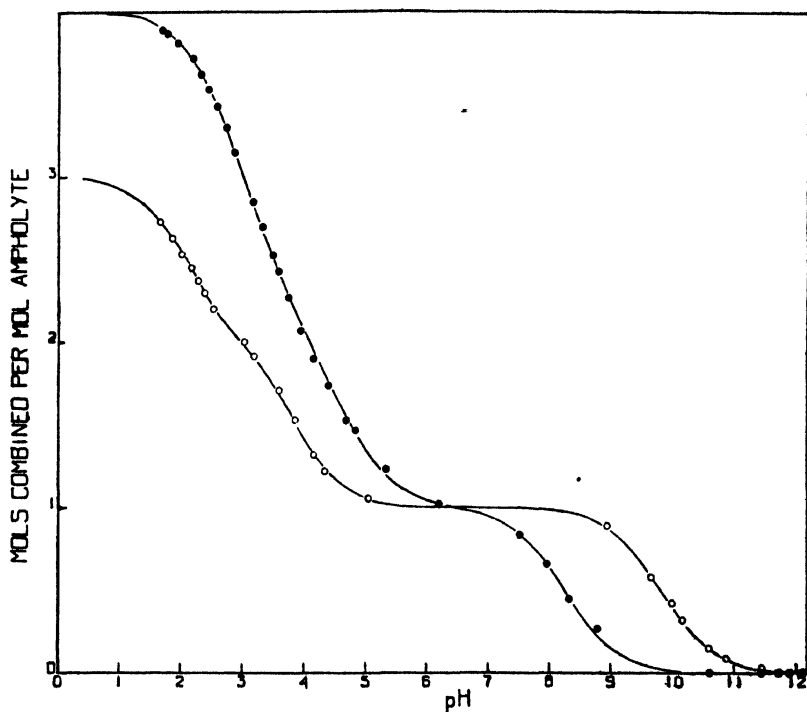


FIG. 2. Curves constructed on the basis of the constants: aspartic acid, $pG_1' = 2.10$, $pG_2' = 3.86$, $pG_3' = 9.82$; aspartyl-aspartic acid, $pG_1' = 2.70$, $pG_2' = 3.40$, $pG_3' = 4.70$, $pG_4' = 8.26$. ● indicates aspartyl-aspartic acid; ○, aspartic acid.

(19). Similar analyses were offered later by Weber (20) and von Muralt (21). In reality the values of the two classes of constants differ by very little and for all practical purposes only the titration constants need be determined. For purposes of comparison

the pK' values of aspartic acid and of aspartyl-aspartic acid are given in a foot-note to Table III.³

In Figs. 1 and 2, the ordinates represent the experimentally found values of x , of the acid or base bound, the abscissæ represent pH. The points are therefore experimental. The curves, however, are those calculated from the titration constants chosen. The experimental points all fall upon the calculated curve to within the accuracy of the measurements.

Values of α corresponding to the respective dissociation stages have been computed (Tables I and II). Their sum should yield the mols of acid or base combined by the ampholyte at each pH value that the experiments reveal, provided the values of pG' were properly chosen.

DISCUSSION

The resemblance of the titration curves of these peptides to those of proteins is very marked. There is the same linear sweep over the greater part of the dissociation range, the same apparent continuous combination in the neighborhood of the isoelectric point. The slope of the peptide curve is likewise steeper than that of the corresponding amino acid and reflects as in the protein, the greater number of dissociating groups.⁴

The dissociation range of the peptides is narrower than that of

³ The peptides were titrated in the presence of a considerable quantity of salt and hence if their true dissociation constants (at zero ionic strength) were sought, the value of K' would be subject to an activity correction. The transformation of K' , the so called apparent constant, to K , the thermodynamically defined constant when all members in the reaction at equilibrium are expressed as activities, consists essentially in multiplying K' by the ratio of the activity coefficients of the charged and uncharged forms of the ampholyte. By working in salt solutions of various concentrations and with a suitable arrangement of the Debye-Hückel equation which permitted them to perform an extrapolation to zero ionic strength, Simms (22) and Bjerrum and Unmack (23) succeeded in evaluating K for certain simple ampholytes.

⁴ Thus if it is assumed that the linear part of the curve may be assigned a single constant, the first differential of the mass action expression yields: $\frac{dx}{dpH} = x(1-x)$, whereby it is apparent that the greater the value of x , the greater the absolute magnitude of the slope.

the amino acids and points to a simultaneous weakening of both acid and basic groups. A list of the apparent dissociation constants is given in Table III, together with the calculated values of the isoelectric points (pI). For the latter, the modified equation of Levene and Simms (24) was employed.

TABLE I
Histidyl-Histidine (0.02 M)

Concentration of HCl or NaOH	m.m.f	pH	p γ	z (experimental)	z (calculated)				Total $\Sigma \alpha = z$
					pG $_1$ ' = 2.25 α_1	pG $_2$ ' = 5.60 α_2	pG $_3$ ' = 6.80 α_3	pG $_4$ ' = 7.80 α_4	
<i>N</i>									
0.080	0.4280	1.56	0.06	0.18	0.170				0.17
0.074	0.4333	1.65		0.21	0.201				0.20
0.067	0.4398	1.76		0.24	0.245				0.24
0.061	0.4479	1.89		0.29	0.304				0.30
0.055	0.4590	2.08		0.38	0.403				0.40
0.049	0.4738	2.33		0.56	0.546				0.55
0.046	0.4841	2.51		0.65	0.646				0.65
0.040	0.5161	3.05		0.84	0.863				0.86
0.037	0.5582	3.76		0.96	0.971	0.014			0.98
0.033	0.6184	4.78		1.16		0.131	0.009		1.14
0.028	0.6648	5.56		1.53		0.477	0.054		1.53
0.024	0.6811	5.84		1.74		0.635	0.090		1.73
0.021	0.6954	6.08		1.90		0.751	0.160	0.018	1.93
0.017	0.7092	6.31		2.10		0.836	0.244	0.031	2.11
0.014	0.7212	6.52		2.26		0.892	0.344	0.049	2.28
0.011	0.7327	6.71		2.43		0.928	0.448	0.075	2.45
0.008	0.7439	6.90	0.22	2.60		0.952	0.557	0.111	2.62
0.006	0.7869	7.63		3.30			0.871	0.404	3.28
0.011	0.8073	7.97		3.57			0.937	0.598	3.54
0.014	0.8321	8.39		3.74			0.975	0.795	3.77
0.018	0.9052	9.63		3.95				0.984	3.98
0.023	1.0093	11.39		4.00				1.00	4.00
0.027	1.0295	11.73		4.00				1.00	4.00
0.031	1.0406	11.92		4.00				1.00	4.00

Table III represents the allocation of the constants to specific groups on the assumption of the *Zwitter Ionen* concept of ampholyte dissociation. From this point of view, in histidyl-histidine, the carboxyl group, dissociating at an acid reaction, would be assigned the value 2.25, the amino group hydrolyzing at an alkali-

line reaction, that of 7.80. In the latter case there occurs the unusual situation of an amino group dissociating at a barely alka-

TABLE II
Aspartyl-Aspartic Acid (0.02 M)

Concentration of HCl or NaOH	E M F.	pH	p γ	α (experimental)	α (calculated)				
					pG ₁ ' = 2.70 α_1	pG ₂ ' = 3.40 α_2	pG ₃ ' = 4.70 α_3	pG ₄ ' = 8.26 α_4	Total $\Sigma \alpha = x$
<i>N</i>									
0.038	0.4369	1.71	0.06	3.89	0.907				3.91
0.035	0.4413	1.78		3.87	0.892	0.977			3.87
0.028	0.4521	1.96		3.81	0.846	0.965			3.81
0.021	0.4665	2.21		3.72	0.756	0.939			3.70
0.017	0.4744	2.34		3.62	0.696	0.919			3.62
0.014	0.4823	2.48		3.53	0.624	0.893			3.52
0.011	0.4904	2.61		3.43	0.551	0.860			3.41
0.008	0.4989	2.76		3.30	0.465	0.814			3.28
0.004	0.5069	2.89		3.15	0.392	0.764	0.985		3.14
0.003	0.5244	3.19	0.22	2.85	0.245	0.619	0.971		2.84
0.006	0.5340	3.35		2.70	0.183	0.529	0.957		2.67
0.009	0.5435	3.51		2.53	0.134	0.437	0.939		2.51
0.011	0.5484	3.59		2.43	0.114	0.392	0.928		2.43
0.014	0.5586	3.77		2.27	0.078	0.299	0.894		2.27
0.018	0.5701	3.96		2.07	0.052	0.216	0.846		2.11
0.021	0.5824	4.17		1.90	0.032	0.145	0.772		1.95
0.024	0.5968	4.41		1.74	0.019	0.089	0.661		1.76
0.028	0.6130	4.69		1.53		0.048	0.506		1.55
0.029	0.6220	4.84		1.47		0.035	0.420		1.46
0.033	0.6513	5.33		1.23		0.011	0.189		1.20
0.036	0.7018	6.19		1.02			0.031	0.991	1.02
0.039	0.7806	7.52		0.84				0.846	0.85
0.042	0.8078	7.98		0.66				0.656	0.66
0.046	0.8285	8.33		0.45				0.460	0.46
0.049	0.8554	8.79		0.27				0.229	0.23
0.052	0.9624	10.60		0.00				0.00	0.00
0.056	1.0119	11.44		0.00				0.00	0.00
0.059	1.0282	11.71		0.00				0.00	0.00
0.064	1.0402	11.91		0.00				0.00	0.00
0.069	1.0493	12.07		0.00				0.00	0.00

line reaction. The imidazole groups would then be assigned the pG' values of 5.60 and of 6.80. It is a task of difficult discrimination to allocate the position in the molecule of either of these

cyclic groups. Referring to the structural formula for histidyl-histidine,

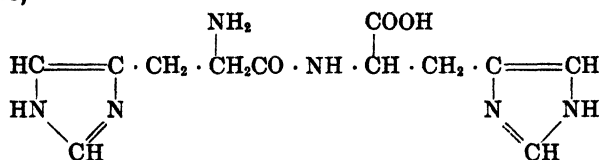


TABLE III*
Titration Constants

	Carboxyl	Imidazole	Amino	Isoelectric point
Histidine†	pG_1' 1.77	pG_2' 6.10	pG_3' 9.18	pI 7.64
Histidyl-histidine	pG_1' 2.25	pG_2' pG_3' 5.60 6.80	pG_4' 7.80	pI 7.31
	Carboxyl		Amino	Isoelectric point
Aspartic acid‡	pG_1' 2.10	pG_2' 3.86	pG_3' 9.82	pI 2.98
Aspartyl-aspartic acid‡	pG_1' 2.70	pG_2' pG_3' 3.40 4.70	pG_4' 8.26	pI 3.04

* The constants are numbered as in the Levene and Simms notation (24) whereby $G_1' > G_2' > G_3'$. . . etc. On the *Zwitter Ionen* concept the carboxylic groups dissociate at acid reactions, the basic groups at alkaline reactions. An apparent exception must be made in the case of histidine where the weakly basic group dissociates at a somewhat acid reaction.

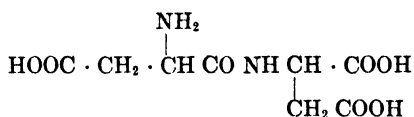
† The pG' values of histidine are similar to those of previous investigators. The results of Schmidt, Appleman, and Kirk (25) at 25° are: $pG_1' = 1.82$, $pG_2' = 6.01$, $pG_3' = 9.17$. Birch and Harris' values at 23° (26) are: $pG_1' = 1.78$, $pG_2' = 5.97$, $pG_3' = 8.97$. For aspartic acid the data of Simms yield (8): $pG_1' = 2.05$, $pG_2' = 3.87$, $pG_3' = 10.00$.

‡ Employing the equations of Simms (19) to calculate the apparent dissociation constants from the values of the titration constants, there is obtained for aspartic acid: $pK_1' = 2.09$, $pK_2' = 3.87$, $pK_3' = 9.82$; for aspartyl-aspartic acid: $pK_1' = 2.62$, $pK_2' = 3.46$, $pK_3' = 4.72$, $pK_4' = 8.26$.

it might be assumed that the imidazole group next to the amino group would be assigned the pG' value 5.60, whereas, that next to the carboxyl, the pG' value 6.80. This assignment is supported by the fact that in all simple peptides (4) the basic and the acid groups are simultaneously weakened. A decrease in the

strength of the amino group would result in an apparent weakening of the neighboring cyclic group; correspondingly a weakening of the carboxyl dissociation would cause a strengthening of the adjacent imidazole group. It is apparent, however, that the presence of the two cyclic groups markedly diminishes the basic properties of the molecule and carries the peptide to a more acid isoelectric point than that of histidine.

In aspartyl-aspartic acid, which possesses three carboxyl groups,



it is still more difficult to assign to each its respective titration constant. Simms (8) found in his studies of glycyl-aspartic acid and aspartyl-glycine, a marked weakening of amino and carboxyl groups. In the former compound, the basic constant was depressed to 8.60 which compares with the pG' value in aspartyl-aspartic acid of 8.26. The decrease in strength of the basic group should greatly strengthen the adjacent carboxyl and give reason to assign the lowest value of pG' , namely 2.70, to this group in the new peptide. This may be confirmed by the assignment of Simm's value of 2.10 for the carboxyl group next to the amino group in aspartyl-glycine. The second carboxyl both in aspartyl-glycine and in glycyl-aspartic acid is around 4.5 and hence the value of 4.70 may be assigned to the corresponding group in aspartyl-aspartic acid, leaving the value of 3.40 for the pG' of the primary carboxyl. The constants of the peptide are more acid than the corresponding pG' of citric acid. It is significant that the introduction of a third carboxyl group into aspartic acid with the subsequent rearrangement, barely shifts the isoelectric point more alkaline than the value of the original amino acid. The rather slightly acid isoelectric points of the prolamins which are so largely composed of dicarboxylic acids, must likewise be due to the mutual weakening effect of their numerous acid groups.

The shift in the dissociation of any particular group in the amino acid, when compared to that same group in the peptide, cannot be formulated on any physical basis without a consideration of the forces of chemical reaction and rearrangement. While

the apparent "weakening" of a group may be due in part to the diminution of forces acting through the chain of atoms, in part it may also be owing to a distortion of the molecular structure so as to bring certain groups near to or farther away from others. The proximity of a carboxyl to a negatively charged carboxyl would, as Ostwald pointed out (27) hinder the former's dissociation; on the other hand, the dissociation of a positively charged basic group would be accelerated. The problem would then, according to Bjerrum (28) be further subdivided into a consideration of the forces propagated through the chain of atoms separating the groups and in estimating the electrostatic effect of one group upon the other. The empirical formulæ heretofore advanced (28, 29) were developed specifically for isomeric acids.

Whatever the theoretical significance of the change in the value of the constants may be, the fact that an imidazole group dissociates at 5.60 as in histidyl-histidine and that of a carboxyl at 4.70 as in aspartyl-aspartic acid, offers what may be an explanation of the presence of such dissociation values in proteins. On the basis of these studies it will be anticipated that the guanidine group of arginine, when the latter is combined in peptide linkage, will be considerably weakened and brought down to the lower alkaline range generally found in the proteins.

I am indebted to Professor E. J. Cohn for suggesting the present series of studies and for his kindness in permitting me to use material from his forthcoming review on the physical chemistry of the proteins. I also wish to thank Dr. A. A. Green and Dr. T. L. McMeekin of this laboratory for their generous interest.

SUMMARY

1. The apparent dissociation constants at 25° have been determined for histidyl-histidine, aspartyl-aspartic acid, histidine, and aspartic acid.

2. It is demonstrated that the introduction of a second imidazole ring in the histidine compound causes a decided weakening of the acid and basic groups as compared with those of histidine itself, accompanied by a shift to a more acid dissociation range of one of the imidazole groups. The isoelectric point of the peptide is more acid than that of the amino acid.

3. The coincidence of three carboxyl groups and one amino group as in aspartyl-aspartic acid results in a mutual weakening of both acid and basic groups and a slightly alkaline shift of the isoelectric point as compared with the isoelectric point of aspartic acid.

4. The titration curves of the peptides studied apparently approach more nearly in character the ordinary titration curve of a protein than any synthetic ampholyte hitherto investigated.

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STUDIES IN THE PHYSICAL CHEMISTRY OF THE PROTEINS

VIII. THE SOLUBILITY OF HEMOGLOBIN IN CONCENTRATED SALT SOLUTIONS. A STUDY OF THE SALTING OUT OF PROTEINS*

By ARDA ALDEN GREEN†

*(From the Department of Physical Chemistry in the Laboratories of
Physiology, Harvard Medical School, Boston)*

(Received for publication, July 1, 1931)

Precipitation by concentrated salt solutions has for long proved the most satisfactory method for the separation of proteins. This procedure was first employed in the middle of the last century by Panum (18), by Virchow (28), and by Bernard (2). The diminution in solubility of proteins in concentrated electrolyte solutions has ever since led to methods for their separation, purification, characterization, and occasionally, classification. The nature of the phenomenon was shown by Hofmeister (13) to depend upon the character of the neutral salt as well as on the protein.

Proteins may be "salted out" by a relatively small increase in electrolyte concentration. Moreover, the effectiveness of concentrated solutions of different salts in the precipitation of proteins varies greatly. It has often been the custom to determine the concentration of salt at which the protein first begins to precipitate, or the concentration at which no more can be induced to precipitate. The small difference in concentration of salt in which most proteins are on the one hand relatively soluble and on the other hand largely precipitated, yielded results which, although rather rough, led to a qualitative description of the phenomenon.

* A preliminary report of a portion of this investigation was read by Cohn and Green (7) at the Twenty-second Annual Meeting of the American Society of Biological Chemists.

† National Research Council Fellow in Medicine, 1927-29, during which time the experiments herein reported were performed.

The decreased solubility of other types of substances in the presence of strong electrolytes must be considered analogous to the precipitation of proteins. In 1892, Setschenow (22) described the decrease in solubility of gases in salt solutions in terms of an exponential equation, empirically derived.

$$S = S_0 e^{kc}, \text{ or } \ln S = \ln S_0 + kc \quad (1)$$

where S_0 is the solubility in water, S the solubility in the electrolyte solution, c the concentration of the electrolyte, and k a constant. The solubility of other non-electrolytes, as well as of gases, in electrolyte solutions has also been studied experimentally. Linderstrøm-Lang (16) has described the solubility of quinone, hydroquinone, and succinic and boracic acids in terms of this equation.

The deviation in solubility in the presence of electrolytes from that expected on the basis of the ideal gas laws is one measure of the "activity coefficient" of the saturating body (15). Debye and MacAuley (9) described the activity coefficients of sugar solutions in the presence of salts in terms of a theoretical equation in which the activity coefficient of the non-electrolyte is, as a first approximation, proportional to the electrolyte concentration. Hückel (14), working on the salting out of electrolytes in electrolyte solutions, added to the original Debye equation an expression in which the variation due to change in the dielectric constant of the solution is also considered a linear function of the concentration. Scatchard (21) has further considered the influence of both electrolytes and non-electrolytes on activity coefficients and Tammann (27) and Randall and Failey (19) have presented and discussed a large amount of the existing data on the solubility of various non-electrolytes in terms of an equation similar to equation (1).

Following the theoretical derivation of the salting out equation for non-electrolytes by Debye and MacAuley and for electrolytes by Hückel, its applicability to the proteins was demonstrated by Cohn (5) who suggested that equations of this type had been found to hold for both strong electrolytes and non-electrolytes, and might be employed in considering the solubility of proteins. He used an equation of the type:

$$\log S = \beta - K_s' \mu \quad (2)$$

in which S is again the solubility, μ is the ionic strength, and β is an intercept constant, which in the case of non-electrolytes is $\log S_0$, and K_s' is the salting out constant. This constant must be considered apparent for certain proteins, since globulins, at least, increase in solubility in dilute salt solutions, and this solvent action considered in subsequent communications, must be considered to be effective to some extent, even in concentrated solutions.

The quantitative data of Chick and Martin (3) on the solubility of egg albumin and of Sørensen (23) on pseudoglobulin and of Sørensen and Høyrup (24) on egg albumin were analyzed by Cohn employing the above relationship in which the logarithm of the solubility was found to be a linear function of the concentration of the ammonium sulfate used as precipitant. That this same exponential relation holds for other proteins in sufficiently concentrated salt solutions has since been reported for hemoglobin by Cohn and Green (7) and for fibrinogen by Florkin (11).

In 1888, Hofmeister (13) studied the effect of protein concentration on the amount of salt necessary for precipitation. Solutions containing a given concentration of protein but varying concentrations of salt were compared, and the salt concentration which would just induce precipitation was noted. This procedure was repeated with other concentrations of protein. When the protein just begins to separate out the solution may be regarded as saturated with respect to that component, and the concentration of the protein to yield its solubility. The protein used was egg white, and the salts, potassium acetate and ammonium sulfate. These data have, because of their historical interest, been recalculated, and are represented in Fig. 1. In the more careful investigations upon chemical individuals that have since been conducted, equilibrium has presumably been more nearly attained, and temperature and acidity more accurately controlled. None the less there can be no doubt that this early investigation beautifully reflects the relations that have subsequently been developed.

Horse hemoglobin has been chosen for the present series of investigations because it is a chemical individual, is readily separated by crystallization, and, being a globulin, can be studied both in dilute and in concentrated salt solutions. Its molecular weight has been determined both by osmotic pressure (1) and by ultracentrifugation (26) methods to be approximately 66,800.

It therefore contains 4 iron atoms and four hematin groups in each molecule. The ultracentrifugation method of Svedberg has also demonstrated that aggregates of molecules are not formed in the presence of 0.019 M phosphate buffers from pH 6.0 to pH 9.05.

Further, Cohn and Prentiss (8) showed that the solubility of oxyhemoglobin was independent of the amount of protein in the solid phase. It dissolved in successive portions of a solvent to the same extent, until the saturating body had been completely dissolved. Carboxyhemoglobin, however, has been used at 25°

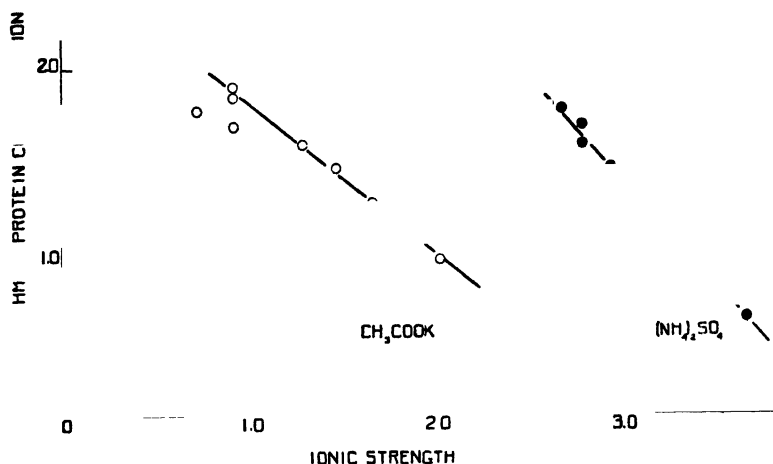


FIG. 1. Curves plotted from the data of Hofmeister (1888)

instead of oxyhemoglobin because of its greater stability at the higher temperature.

The solubility of crystalline horse hemoglobin in concentrated solutions of various salts has now been studied at constant temperature and pH and also in a given salt under varying conditions. The experimental results may be thus conveniently divided into two parts. The first deals with the change of solubility of oxy- and carboxyhemoglobin in phosphate buffer mixtures at varying temperature and acidity. The salting out constant, K_s' , in a given salt appears to be independent of pH and also of temperature, and therefore to yield a physical constant characteristic only of the protein and the salt. The variation of K_s' with the

salt, that is to say, the quantitative description of the phenomenon noted by Hofmeister, constitutes the second part of this communication.

Experimental Method

Crystalline horse hemoglobin was used in this series of experiments. It was prepared by the method developed in this laboratory (10). After the cells in citrated horse blood had been allowed to settle the plasma was decanted. The cells, 3 or 4 liters in quantity, were then suspended in 12 liters of ice-cold hypertonic salt solution. This was either 1.5 per cent NaCl or 1.8 per cent KCl. The hemoglobin was separated from this solution by means of the Sharples centrifuge. If the filtrate was too concentrated and crystallization began immediately, water was added. The hemoglobin was always retained in the cold room as much as possible.

Crystallization was effected by the addition of 0.1 N acid which was added drop by drop, while the hemoglobin was thoroughly stirred mechanically in order to prevent a local excess. 0.1 N sulfuric acid was used in preparing hemoglobin for the sulfate experiments and 0.1 M phosphoric acid for the phosphate experiments. The hemoglobin for the citrate experiment was prepared with acetic acid since the solubility of hemoglobin in sodium acetate was being studied on the same preparation.

The strongly acid and alkaline solutions used in the preparation of hemoglobin must be employed with great circumspection. If added directly to the protein without precautions as to the method of addition, denaturation would promptly result. If, however, they are added very slowly, and the solution is at the same time effectively stirred so that no local excess of acid or base occurs, there is no denaturation.

The onset of crystallization could usually be detected by the development of a sheen in the solution, unless the solution was too dilute or the hemoglobin not completely oxygenated. It was generally necessary to add about 150 cc. of 0.1 N acid for every liter of the original cells. This brought the hemoglobin into the neighborhood of its isoelectric point where crystallization should take place. If crystallization appeared to be unsuccessful a drop of the solution was placed upon a microscope slide and allowed

to dry slightly. If the crystals so formed were found to be needles, the solution was still too alkaline. Horse hemoglobin crystallizes in rhomboidal plates in the neighborhood of pH 6.6, in needles at more alkaline reactions. Care was always taken not to add too much acid since hemoglobin is so readily destroyed.

Recrystallization was effected at least once and usually twice. This was accomplished by first carefully dissolving the hemoglobin by the addition of the smallest requisite amount of normal alkali. The alkali was added in the same careful manner as the acid. The hemoglobin was then recrystallized by the addition of an equivalent amount of 0.1 N acid. Either sodium or potassium hydroxide was used to dissolve the protein, depending on whether the salt used was one of sodium or of potassium. Sodium hydroxide was used in preparing the material for the magnesium sulfate experiment. After each crystallization the hemoglobin crystals were washed two or three times in distilled water. This was accomplished by centrifuging in the ordinary centrifuge and decanting the supernatant solution, adding a quantity of water equal to about one-third the volume of the centrifuged crystals, stirring thoroughly, and again centrifuging. The crystals were then dissolved in alkali and the solution centrifuged to remove any remaining cell debris. At this point the hemoglobin was thoroughly saturated with carbon monoxide if carboxyhemoglobin was to be used in the experiment. Acid was then added to effect recrystallization.

In most cases the last precipitation was accomplished in concentrated solutions of the electrolyte with which the experiment was concerned. The last crystallization was watched carefully, and if the concentration of hemoglobin was not too high, crystallization did not take place immediately upon neutralization of the alkali used in dissolving the crystals, and it was possible to transfer portions of the solution to vessels containing an amount of very concentrated electrolyte solution sufficient to yield the approximate salt concentration desired in the experiment. If the hemoglobin did crystallize in the dilute salt solution, the concentrated solution was added as soon as possible. Crystallization of the hemoglobin in an electrolyte solution of the approximate composition of that in which solubility was to be determined facilitated the attainment of equilibrium.

The solubility determinations were made in the manner previously described from this laboratory (8). The crystals were repeatedly equilibrated with a given salt solution at constant temperature, filtered, and the filtrate analyzed for nitrogen. All determinations were made as near pH 6.6 as possible, since this is the point of minimum solubility. The relative amounts of the mono- and dihydrogen phosphates necessary were calculated by the equation of Cohn (6). Since concentrations greater than those previously studied were also employed, the pH of the mixtures was checked with the hydrogen electrode. The pH of the saturated solutions of carboxyhemoglobin in the other electrolytes used was controlled by adjusting the pH of both the hemoglobin and the salt solution. When the solution was well saturated with carbon monoxide reproducible E.M.F. measurements could be made with the hydrogen electrode. The satisfactory use of the hydrogen electrode for carboxyhemoglobin has also been reported by Hastings *et al.* (12) and by Stadie and Hawes (25). The pH of the saturated solutions is recorded in Table V. In general it remains between pH 6.5 and 6.7, a range in which solubility changes but little.

In determining the solubility of carboxyhemoglobin at 25° the crystals were placed in closed 250 cc. centrifuge bottles. The container was originally about half filled with crystals. The appropriate electrolyte solution was added and the mixture repeatedly and thoroughly saturated with carbon monoxide, since reduced hemoglobin is much more soluble than either oxyhemoglobin or carboxyhemoglobin. The containers were gently shaken for at least 5 hours in a constant temperature water bath at 25°. This period had been previously determined to be sufficient in which to attain equilibrium. The shaking apparatus was that previously described (4). After the solution had become saturated it was filtered at 25° through a Whatman No. 42 filter paper and the filtrate was analyzed. The crystals were returned to their container, more electrolyte solution was added, the mixture was resaturated with carbon monoxide and again equilibrated at 25°.

The oxyhemoglobin was brought into equilibrium with the phosphate buffer solutions, with use of the method of Cohn and Prentiss (8), by placing the hemoglobin in open vessels immersed

in an ice water bath and stirring by rotating rods projecting into the cups. The use of open containers insures constant saturation of the hemoglobin with oxygen from the air. These solutions were also filtered through funnels immersed in an ice water bath. The carboxyhemoglobin solutions at 0° were, however, equilibrated in closed vessels and a different shaking machine was used. These solutions were also filtered at 0°.

In all cases the crystals were washed with the salt solutions until the solubility of successive filtrates became constant. Solubility was calculated from the nitrogen analyses, by assuming horse hemoglobin to contain 17.7 per cent nitrogen.

Part I. Solubility of Hemoglobin in Concentrated Solutions of Phosphate

In order adequately to characterize solubility in concentrated electrolyte solutions the variation in β and K_s' , the constants in equation (2), have been estimated under different conditions.

The solubility of carboxyhemoglobin in potassium phosphate buffers at 25° and pH 6.6 is described by the data given in Table I. The results are given in detail in order to illustrate the constancy of solubility in successive filtrates and in different preparations, provided temperature and acidity are maintained constant. The solubility of carboxyhemoglobin and of oxyhemoglobin at 0°, and at the same pH is given in Table II. In order to conserve space the number of successive equilibrations and the average of the measurements in each experiment are published. The detailed protocols comparable to Table I for all other experiments reported in these papers are filed in the laboratory. In all tables the experiment number is given in the first column, experiments of the same number being upon the same preparation of hemoglobin. The experiments on oxyhemoglobin and on carboxyhemoglobin belong to different series.

When equilibrium was established it was assumed that the hemoglobin-salt solution contained the same amount of salt as the original electrolyte solution. Thus, in estimating the ionic strength the amount of water present per liter of solution saturated with hemoglobin was calculated by subtracting the weight of the hemoglobin, plus the weight of the salt in the original electrolyte solution, from the total weight of the solution. Calculations in

TABLE 1

Solubility of Carboxyhemoglobin at 25° in Phosphate Solutions at pH 6.6

Experiment No.	Filtrate No.	Concentration phosphate	Mol fraction K_2HPO_4	Density	ϵ_{12}	μ	Solubility			Log solubility per 1000 gm. H_2O
							Hb per liter	Hb per 1000 gm. H_2O	Mol fraction Hb $\times 10^6$	
		<i>mols per l.</i>					<i>gm.</i>	<i>gm.</i>		
4	3						6.72			
	4						6.61			
	5						6.44			
	6						6.61			
Average...		1.072	0.526	1.1239	2.20	2.32	6.59	6.94	1.84	0.841
3	3						2.69			
	4						2.53			
	5						2.56			
	6						2.58			
Average...		1.267	0.527	1.1453	2.60	2.76	2.59	2.74	0.723	0.438
5	4						2.50			
	5						2.57			
	6						2.57			
	7						2.60			
Average...		1.267	0.527	1.1434	2.60	2.76	2.56	2.71	0.715	0.433
3	3						1.75			
	4						1.58			
	5						1.63			
	6						1.60			
Average...		1.367	0.527	1.1574	2.80	2.97	1.64	1.74	0.458	0.240
3	3						1.07			
	4						1.05			
	5						1.02			
	6						1.015			
Average...		1.462	0.527	1.1658	3.00	3.20	1.04	1.11	0.292	0.045

TABLE I—*Concluded*

Experiment No.	Filtrate No.	Concentration phosphate	Mol fraction K_2HPO_4	Density	Γ	μ	Solubility			Log solubility per 1000 gm. H_2O
							Hb per liter	Hb per 1000 gm. H_2O	Mol fraction Hb $\times 10^6$	
4	5	<i>mols per l.</i>					<i>gm.</i>	<i>gm.</i>		
	6						1.09			
	6						1.06			
	7						1.00			
Average..		1.462	0.527	1.1646	3.00	3.20	1.05	1.12	0.294	0.049
3	3						0.441			
	4						0.367			
	5						0.367			
	6						0.378			
Average..		1.657	0.527	1.1830	3.40	3.68	0.388	0.418	0.109	1.621
4	3						0.446			
	4						0.463			
	5						0.429			
	6						0.429			
Average...		1.657	0.527	1.1843	3.40	3.68	0.438	0.475	0.124	1.677

which the weight of hemoglobin was subtracted from the total weight of the solution and the remainder assumed to contain the same proportion of salt to water as in the original buffer yielded the same result. Analysis of the composition of salt solutions of varying nature and concentration containing hemoglobin, indicated that 1.4 gm. of hemoglobin displaced 1 gm. of water. Calculations could have been made using this value, but because of the relatively low amount of hemoglobin, all the methods of calculation yield approximately the same result. The symbol Γ is that introduced by Debye for the "ionic strength" per liter; while μ is rigorously employed as defined by Lewis ((15) p. 373), being calculated as the ionic strength per 1000 gm. of water. The solubility of the hemoglobin was experimentally determined in

gm. per liter. The results have been calculated in terms of gm. of solute per 1000 gm. of water and also in terms of the mol fraction, N , the molecular weight of horse hemoglobin being assumed to be 66,800. Both of these functions of solubility should be independent of volume changes due to temperature.

TABLE II

Solubility of Hemoglobin in Concentrated Phosphate Solutions at pH 6.6

Experiment No	No. of determinations	Concentration phosphate	Mol fraction K_2HPO_4	Density	μ	Solubility			Log solubility per 1000 gm. H_2O
						Hb per liter	Hb per 1000 gm H_2O	Mol fraction $Hb \times 10^5$	

Solubility of carboxyhemoglobin at 0°

		<i>mols per l.</i>				<i>gm.</i>	<i>gm.</i>		
3	3	0 879	0.524	1.1093	1.98	68.7	75.5	2.00	1.878
4	3	1 072	0.526	1.1257	2.41	52.8	57.9	1.53	1.762
3	3	1.267	0.527	1.1444	2.81	22.6	24.3	0.640	1.386
5	5	1.267	0.527	1.1427	2.81	22.8	24.5	0.645	1.389
3	4	1.462	0.527	1.1629	3.24	9.56	10.3	0.271	1.012
4	3	1.462	0.527	1.1627	3.24	12.08	13.0	0.342	1.114
3	4	1.657	0.527	1.1826	3.69	3.62	3.93	0.103	0.594

Solubility of oxyhemoglobin at 0°

		<i>mols per l.</i>				<i>gm.</i>	<i>gm.</i>		
16	4	0 879	0.524	1.1063	1.95	52.2	56.6	1.498	1.753
16	3	1 072	0.526	1.1240	2.38	34.7	37.4	0.990	1.573
16	4	1.267	0.527	1.1426	2.80	18.8	20.3	0.534	1.307
17	5	1.267	0.527	1.1429	2.80	19.3	20.8	0.547	1.318
17	4	1.367	0.527	1.1526	3.01	12.8	13.77	0.362	1.139
17	3	1.560	0.527	1.1726	3.46	5.07	5.48	0.144	0.739
17	3	1.657	0.527	1.1815	3.70	3.41	3.71	0.097	0.569
17	5	1.757	0.527	1.1925	3.94	1.84	2.01	0.052	0.303

Variation of Solubility with Temperature—The values of β and K_s' in equation (2), at constant pH, namely 6.6, have been calculated, by means of simultaneous equations, for carboxyhemoglobin at 0° and at 25° and are given in Table III. It is to be noted that the calculated values of K_s' are remarkably constant for the different salt concentrations in both cases and have the same

value, 0.88, at both temperatures. In Fig. 2, where the logarithm of the solubility is plotted against the ionic strength, the conformity of the experimental points with the straight line demanded by the equation is evident. Moreover the lines are drawn parallel.

Whereas K_s' thus appears to be independent of temperature the intercept constant, β , varies widely; for carboxyhemoglobin at 0° it is 3.86, and at 25° 2.87, a difference of 1.0. Since β is a logarithmic function this means that in concentrated salt solutions of the same ionic strength horse hemoglobin is 10 times more soluble at 0° than at 25°. This is an inversion of the solubility relations in dilute solutions where increase in temperature increases the solu-

TABLE III

Values of β and K_s' for Hemoglobin in Concentrated Phosphate Solutions at pH 6.6

Oxyhemoglobin at 0°			Carboxyhemoglobin at 0°			Carboxyhemoglobin at 25°		
μ	K_s'	β	μ	K_s'	β	μ	K_s'	β
2.80	0.864	3.78	2.41	0.864	3.85	2.32	0.908	2.88
3.01	0.867	3.79	2.81	0.878	3.89	2.76	0.901	2.87
3.46	0.846	3.78	3.24	0.891	3.86	2.97	0.886	2.85
3.70	0.869	3.83	3.69	0.906	3.84	3.20	0.869	2.88
3.94	0.953	3.77				3.68	0.854	2.87
Average..	0.879	3.79		0.885	3.86		0.884	2.87

bility. This increased solubility of proteins in concentrated solutions of salt in the cold has been used by Redfield¹ as a means of crystallizing hemocyanin, and by the author in crystallizing human hemoglobin. The effect of temperature changes on solubility of proteins had of course been used before in their crystallization. Ritthausen (20), Osborne (17), and subsequent investigators have crystallized vegetable globulins by heating them gently in salt solutions and then gradually cooling. At the concentrations of salt employed in this general method of crystallization, edestin, like hemoglobin, is in the range of maximum solubility. Here temperature has little effect upon hemoglobin,

¹ Personal communication.

whereas solubility is increased by temperature at lower, but decreased at higher concentrations of salt.

The decreased solubility of certain non-electrolytes, for instance gases, at higher temperatures has long been known. The data for the solubility of oxygen and nitrous oxide recalculated by Randall and Failey (19) yields the same value for K_s' in a given salt solution at different temperatures.

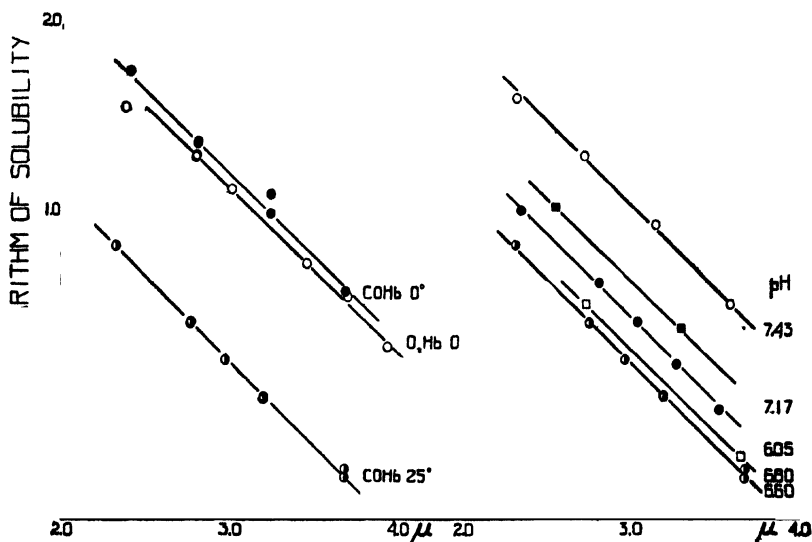


FIG. 2. The solubility of hemoglobin in concentrated phosphate buffers, of varying temperature and pH.

The numerical values of K_s' are, of course, dependent upon the units in which the concentration of hemoglobin and of salt is defined. If solubility is calculated in gm. of protein per liter of solution and the salt concentration as $\frac{\Gamma}{2}$; that is, if equation (2) has the form

$$\log S_{(\text{gm. per liter})} = \beta - K_s' \frac{\Gamma}{2} \quad (3)$$

K_s' is 1.00 for carboxyhemoglobin at 25° and pH 6.6 and β is 3.01. If, on the other hand, solubility is calculated as mol fraction, N , and the equation is written

$$\log N = \beta - K_s' \frac{\Gamma}{2} \quad (4)$$

K_s' is 1.01. All of these equations describe the experimental findings over the range of salt concentration investigated. The convention has been adopted generally of describing solubility in terms of gm. of protein per 1000 gm. of water and ionic strength as μ , in characterizing the solubility of protein in concentrated salt solutions.

TABLE IV

Sørensen and Høyrup's Experiments upon the Solubility of Egg Albumin in Ammonium Sulfate at Various Temperatures*

Experiment No.	Filtrate No.	Temperature	μ	Solubility	Log S	β
		°C.				
1	6	0	6 408	3.18	0.502	6.333
6	7	0	5 457	21 81	1 339	6 305
8	6	0	5 457	22 02	1 343	6 309
9	7	0	5 457	19 90	1 299	6 265
11	6	0	5.457	21 30	1 328	6.294
12	7	0	5 457	20 71	1 316	6 282
4	4	12	6 408	2 09	0 320	6.151
5	4	12	5.457	14 89	1.173	6.139
7	6	20	6 408	1 81	0 258	6 089
8	8	20	5.457	13 70	1.137	6 103
10	6	29	6 408	2 24	0 350	6 185
5	6	29	5.457	16 80	1.225	6.191
11	4	29	5.457	16 00	1 204	6.170

* See (24) p. 234, Table 43.

The values of K_s' and β for oxyhemoglobin at 0° also have been calculated and are recorded in Table III. K_s' has the same value as for carboxyhemoglobin at the same temperature and pH. The difference in β is slight, being 3.79 rather than 3.86. The solubility curves are therefore almost superimposable in concentrated salt solutions as may also be seen in Fig. 2. This is not the case in more dilute salt solutions where carboxyhemoglobin is less soluble than oxyhemoglobin.

Sørensen and Høyrup studied the rate of precipitation of egg albumin at various temperatures (24). Equilibrium was reached

more rapidly in systems which were changed from a condition of lower to one of higher solubility. The lowest solubilities recorded, representing the most complete crystallization of the protein are reproduced in Table IV. Two concentrations of ammonium sulfate were employed. On the assumption that K_s' is independent of temperature, values of β should be independent of salt concentration, but dependent upon temperature precisely as they have been shown to be dependent upon pH (5). K_s' for egg albumin in ammonium sulfate when the salt concentration is employed as ionic strength per liter, is 1.19 (7); when mols per

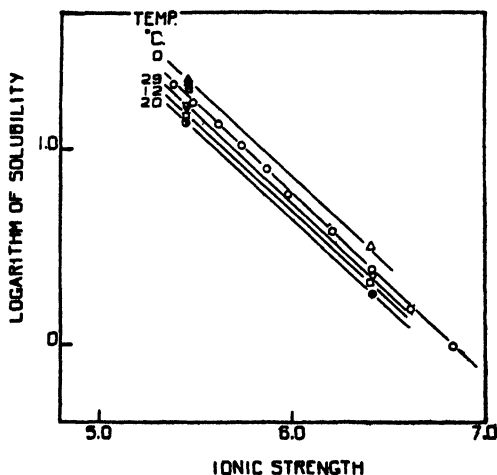


FIG. 3. The solubility of egg albumin at various temperatures from the experiments of Sørensen and Høyrup (24).

1000 gm. of water, 0.91 (11). Values of β , calculated on the latter basis, are given in Table IV. These appear to be consistent with the hypothesis of a constant value of K_s' as may be seen in the accompanying Fig. 3, where the lines through the experimental points are again drawn parallel. A series of experiments carried out at room temperature by the same authors is represented in Fig. 3 and recorded in their Table 40 ((24) p. 224). As in hemoglobin, solubility at 0° is greater than solubility at room temperature in concentrated solutions of electrolytes, but egg albumin exhibits a minimum solubility at about 25°. Solubility determi-

nations have not been carried out on hemoglobin at enough different temperatures to ascertain if this is also the case for that protein. However, in egg albumin as in carboxyhemoglobin K_s' , in contrast to β , is apparently independent of temperature.

Variation of Solubility with pH—The solubility of carboxyhemoglobin at 25° in phosphate buffers at various reactions on both sides of the pH of minimum solubility, is also represented in Fig. 2. Here also the lines are parallel to that at pH 6.6 under the same conditions. Data defining the variation of solubility in different concentrations of salt over a considerable pH range are considered in the next communication of this series. Here it seems necessary merely to indicate the constancy of K_s' . Since there is such a change in β with pH, and since the solubility is increased ten-fold in changing from pH 6.6 to 7.4, the necessity of controlling pH in the salting out of proteins is evident.

Sørensen and Høyrup's data on the solubility of egg albumin in $(\text{NH}_4)_2\text{SO}_4$ at varying pH values, and in two concentrations of salt, have previously been shown by Cohn (5) to yield constant values for β at a given pH when K_s' is taken as a constant. The constancy of K_s' was also demonstrated by Florkin in a study from this laboratory upon the solubility of fibrinogen. On the other hand, β varies with pH as in egg albumin and in hemoglobin.

The investigations on these three proteins may be considered to have demonstrated that although the solubility of a protein in a given salt solution varies with temperature and pH, K_s' remains constant. Accordingly in the next section of this paper these conditions are maintained constant and the variation of K_s' with the nature of the salt is considered.

Part II. Solubility of Hemoglobin in Concentrated Solutions of Different Salts. The Hofmeister Series

The relative effectiveness of the various ions in the precipitation of a protein under constant conditions can be quantitatively expressed by the value of the apparent salting out constant, K_s' . The value of β , the intercept constant, appears to be approximately the same for a given protein at a given pH and temperature. K_s' on the other hand is the slope constant, and varies widely with the electrolyte. Evaluation of β and K_s' for different electrolytes should be an accurate description of the precipitating power of these electrolytes on the given protein.

TABLE V
Solubility of Carbozyhemoglobin at 25°

Experiment No.	No. of determinations	Concentration electrolyte	Density	μ	pH	Solubility			Log solubility per 1000 gm. H ₂ O
						Hb per liter	Hb per 1000 gm. H ₂ O	Mol fraction Hb $\times 10^4$	
Solubility in sodium sulfate									
		<i>mols per l.</i>				<i>gm.</i>	<i>gm.</i>		
11	5	0.675	1.0816	2.12	6.60	9.56	9.78	2.609	0.990
15	3	0.80		2.46	6.71	5.82	5.94	1.576	0.774
11	5	0.809	1.0962	2.48	6.50	4.74	4.87	1.296	0.688
11	4	0.944		2.91	6.28	2.39	2.46	0.651	0.391
15	1	1.00		3.09	6.70	1.92	1.98	0.524	0.297
24	1	1.065	1.1243	3.30	6.57	1.305	1.345	0.356	0.129
11	4	1.079		3.34	6.61	1.15	1.29	0.343	0.111
11	4	1.214	1.1405	3.76	6.48	0.552	0.577	0.152	1.761
Solubility in ammonium sulfate									
6	4	1.20	1.0821	3.90	6.63	3.30	3.59	0.946	0.555
21	1	1.24	1.0904	4.03	6.60	2.26	2.45	0.645	0.389
21	1	1.36		4.48	6.40	1.20	1.32	0.346	0.121
6	5	1.40	1.0950	4.64		1.41	1.565	0.410	0.194
6	5	1.48	1.0992	4.92	6.50	0.922	1.023	0.268	0.009
6	6	1.60	1.1074	5.38	6.52	0.535	0.599	0.157	1.777
6	6	1.80	1.1202	6.12	6.48	0.193	0.2185	0.0568	1.3395
6	5	2.00	1.1320	6.92	6.56	0.063	0.0727	0.0188	2.8615
Solubility in magnesium sulfate									
11	4	1.940	1.2175	7.93	6.34	4.75	4.86	1.267	0.687
21	1	2.04		8.43	6.30	4.63	4.75	1.240	0.677
11	3	2.183		8.99	6.28	1.98	2.03	0.526	0.308
11	4	2.304	1.2529	9.47	6.33	1.55	1.595	0.405	0.203
11	3	2.425	1.2607	10.00	6.24	1.00	1.03	0.266	0.013
Solubility in sodium citrate									
7	4	0.404	1.0852	2.50	6.67	8.38	8.62	2.30	0.935
7	4	0.505	1.1033	3.13		3.33	3.44	0.918	0.536
7	4	0.606	1.1227	3.77		1.28	1.33	0.352	0.124
7	4	0.808	1.1615	5.09	6.54	0.172	0.181	0.048	1.256

In Table V are recorded solubility determinations on carboxyhemoglobin at 25° and pH 6.6 in concentrated solutions of sodium, ammonium, and magnesium sulfates and in sodium citrate. The column headings have the same significance as those in Table I. These results are graphically represented (Fig. 4) in order to show that the above linear relation holds, and that K_s' varies for the different salts.

The values for K_s' and β have been calculated by means of simultaneous equations and the average values reported in

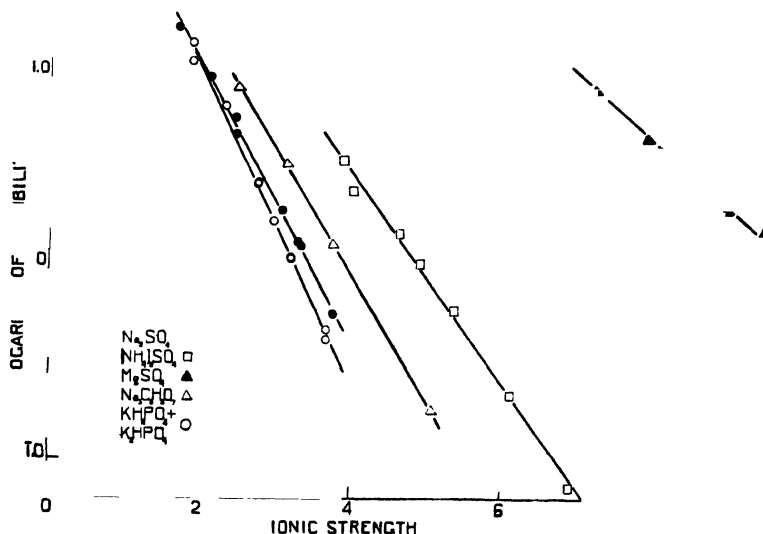


FIG. 4. The solubility of carboxyhemoglobin at 25° and pH 6.6 in concentrated solutions of various electrolytes.

Table VI on the basis of equations (2), (3), and (4). The order of decreasing precipitating ability and, also, the order of decreasing K_s' is $\text{KH}_2\text{PO}_4 + \text{K}_2\text{HPO}_4$, Na_2SO_4 , $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, $(\text{NH}_4)_2\text{SO}_4$, and MgSO_4 . The chlorides studied, and considered elsewhere, have even less precipitating ability, but may be added in the order KCl , NaCl . If one calculated K_s' in terms of equivalents rather than on an ionic strength basis, the following values are obtained: $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, 1.29; $\text{KH}_2\text{PO}_4 + \text{K}_2\text{HPO}_4$, 1.15; Na_2SO_4 , 1.08; $(\text{NH}_4)_2\text{SO}_4$, 0.84; MgSO_4 , 0.62. In the series developed by

Hofmeister the above salts, on the basis of equivalents per liter, are given in the following order: Na_2SO_4 , K_2HPO_4 , and $\text{Na}_2\text{C}_4\text{H}_4\text{O}_7$, $(\text{NH}_4)_2\text{SO}_4$, MgSO_4 , NaCl , and KCl (13). This was, as we have seen, determined as the lowest concentration of salt necessary to cause precipitation of the protein. The agreement is striking.

The values of β , recorded in Table VI, are not constant. Since β is a hypothetical extrapolated quantity, this is not surprising. Hemoglobin is a globulin and the linear relation holds only in concentrated solutions of salts. The variations in β may thus be

TABLE VI

Values of β and K_s' for Carboxyhemoglobin in Concentrated Salt Solutions at pH 6.6

Electrolyte	$\text{KH}_2\text{PO}_4 + \text{K}_2\text{HPO}_4$	Na_2SO_4	$\text{Na}_2\text{C}_4\text{H}_4\text{O}_7$	$(\text{NH}_4)_2\text{SO}_4$	MgSO_4
Defined by the equation, $\log S_{(\text{per } 1000 \text{ gm. H}_2\text{O})} = \beta - K_s' \mu$					
β	2.87	2.51	2.55	2.80	3.13
K_s'	0.88	0.721	0.65	0.57	0.31
Defined by the equation, $\log S_{(\text{per liter})} = \beta - K_s' \frac{\Gamma}{2}$					
β	3.01	2.53	2.60	3.09	3.23
K_s'	1.00	0.76	0.69	0.71	0.33
Defined by the equation, $\log N = \beta - K_s' \frac{\Gamma}{2}$					
β	4.49	5.90	4.04	4.49	4.71
K_s'	1.01	0.73	0.69	0.71	0.34

explained by the increased solubility in the presence of lower concentrations of electrolytes. This effect will be further considered in a later communication.

Fibrinogen has been quantitatively studied in sodium chloride, ammonium sulfate, and in phosphate buffers by Florkin (11). Fibrinogen is completely precipitated by sodium chloride whereas the solubility of hemoglobin is not at all decreased in a saturated solution of the same salt. The salts are, however, effective as precipitants in the same order in fibrinogen as in hemoglobin.

The rôle of the protein in this relation is graphically represented in Fig. 5 in which is plotted the solubility of a number of proteins in ammonium sulfate. The curves for pseudoglobulin and egg albumin are from the data of Sørensen (23) and of Sørensen and Høyrup (24) also recalculated by Cohn (5). The curves for fibrinogen are Florkin's.

The great difference in the slopes of the straight lines and therefore in the values of K_s' for different proteins is to be stressed rather than their position, for neither was the pH of the systems

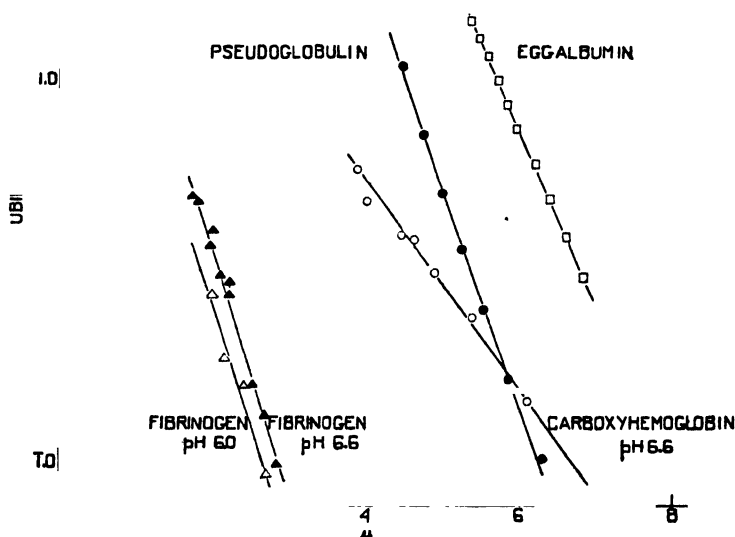


FIG. 5. The solubility of various proteins in concentrated ammonium sulfate solutions.

studied always the same, nor were all the proteins near their isoelectric points, where solubility might be expected to be minimal.

Studies of the variation of β under different conditions may be expected to indicate procedures for the separation of proteins from each other that have not yet been apprehended, but the value of K_s' will presumably not be found to vary widely with physicochemical conditions, but rather to reflect a characteristic of the protein more deep seated than its state. In this connection it should be noted that the solubility of human and horse hemoglobin under identical conditions differs enormously. This

suggests both the lack of wisdom of inferences as to solubility relationships of analogous proteins in different species, and the delicacy of the methods of analyses employed in this method of characterization.

I am indebted to Professor Edwin J. Cohn for suggesting these problems on the solubility of hemoglobin and for his continued interest during the progress of the investigation.

SUMMARY

1. The solubility of carboxyhemoglobin and of oxyhemoglobin has been studied in concentrated potassium phosphate buffers, and of carboxyhemoglobin in concentrated solutions of sodium, ammonium, and magnesium sulfates and sodium citrate.

2. The solubility in concentrated salt solutions at constant pH and temperature is described by equations of the type:

$$\log S = \beta - K_s' \mu$$

3. β is a variable dependent upon the nature of the protein and the pH. Increase in temperature from 0° to 25° decreases ten-fold the solubility of carboxyhemoglobin in concentrated phosphate solutions.

4. K_s' appears to be independent of temperature and of pH.

5. The value of K_s' varies with the salt, and decreases in the order: $\text{KH}_2\text{PO}_4 + \text{K}_2\text{HPO}_4$, Na_2SO_4 , $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, $(\text{NH}_4)_2\text{SO}_4$, and MgSO_4 . This is essentially the same order as that given by Hofmeister in 1888.

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STUDIES IN THE PHYSICAL CHEMISTRY OF THE PROTEINS

IX. THE EFFECT OF ELECTROLYTES ON THE SOLUBILITY OF HEMOGLOBIN IN SOLUTIONS OF VARYING HYDROGEN ION ACTIVITY WITH A NOTE ON THE COMPARABLE BEHAVIOR OF CASEIN*

By ARDA ALDEN GREEN†

*(From the Department of Physical Chemistry in the Laboratories of
Physiology, Harvard Medical School, Boston)*

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INTRODUCTION

The evaluation of constants describing the dissociation of hemoglobin as an acid was first attempted because of its physiological significance. This followed the recognition by Parsons (23) and by Henderson (14) that the chemical equilibrium between hemoglobin, oxygen, and carbonic acid depended upon the difference in the strength of the acid dissociation constants of reduced and oxyhemoglobin. The dissociation of hemoglobin has since been the subject of extensive investigations by Van Slyke, Hastings, Stadie, and others (12, 13, 30) by electrometric and gasometric methods.

Dissociation constants may be calculated from solubility measurements if the increase in solubility following the addition of acid or alkali be assumed to depend upon the appearance of soluble ionized forms in addition to the component in equilibrium with the solid phase. The ampholyte equilibria involved have been discussed by Michaelis (20) and by Sørensen (26). Solubility measurements were employed in the determination of the apparent acid dissociation constants of casein by Cohn and Hen-

* A preliminary report of some of the material presented in this paper was given at the Twenty-second Annual Meeting of the American Society of Biological Chemists (5).

† National Research Council Fellow in Medicine, 1927-29, during which time this investigation was in progress.

dry (6). Since then the method has been applied in the study of both acid and basic constants of tyrosine by Hitchcock (15), and of other amino acids by Cannan and Knight (2), by Sano (24), and by Dalton, Kirk, and Schmidt (8). The analysis of the solubility of hemoglobin on a similar basis has been attempted in this communication.

Electrolytes profoundly influence dissociation. Dissociation constants determined in the presence of neutral salts, even in the presence of an appreciable amount of the substance investigated, are therefore apparent. The true dissociation constants are related to the apparent values experimentally determined in terms of activity coefficients, and are generally estimated by an extrapolation, either mathematical or graphical. Although the validity of the dissociation constants depends upon the equation employed in the extrapolation, behavior in terms of apparent dissociation constants is independent of theory.

Solubility measurements in salt solutions yield activity coefficients as well as dissociation constants. Equations in terms of which solubility is quantitatively described must therefore contain terms defining not only change in dissociation with change in hydrogen ion activity, but also change in activity coefficient with ionic strength. Numerous investigators (4, 16, 25, 28) have considered dissociation constants derived from electromotive force measurements at various ionic strengths, often in terms of the Debye-Hückel theory of strong electrolytes. Solubility measurements upon proteins in salt solutions of varying hydrogen ion activity are considered in this communication.

THEORETICAL

The simplest assumption on the basis of which the solubility of hemoglobin may be interpreted is that in its saturated solutions it is in equilibrium with but one solid component. On this assumption variation of solubility with pH must depend upon the formation of different ionic species. Thus, in combining with base, hemoglobin may be considered as a polyvalent acid containing n hydrogen atoms dissociating to give H_nHb , $H_{n-1}Hb^-$, . . . Hb^{n-} . The activities of each of these may be related to the others by means of the mass law. If the saturating body be assumed to be any one of these species, the amount of the others present at known hydrogen ion activities can be calculated.

The solubility of casein in systems containing small amounts of sodium hydroxide was adequately described by Cohn and Hendry (6) in terms of the equation for a divalent acid. Thus, despite the large number of dissociable hydrogen atoms, casein behaved as though it contained but two.

"The solubility of proteins in systems containing sodium hydroxide yield not only the dissociation constants of the protein, . . . but also the equivalent combining weight of the protein. . . . The equivalent combining weight of casein as estimated by this method varied from 2,096 to 2,166 grams per mol sodium hydroxide. . . . Since the probable molecular weight of casein is 192,000, approximately 90 acid groups in the casein molecule must be involved in its passage into solution. It is extraordinary that despite this large number of groups, casein appears to behave as though it were a divalent acid" (3) p. 407).

Egg albumin and hemoglobin in concentrated salt solutions also behave as though they were divalent acids (5). The solubility of all of these proteins may be described if it is assumed that the concentration of the ionic species formed by the dissociation of 1 hydrogen atom is negligible and that the only negatively charged ionic form present has resulted from the apparent dissociation of 2 hydrogen atoms.

The ability of a protein to combine with acid is dependent upon its polyvalent basic character, and the possible ionic species are $\text{Hb}(\text{OH})_n$, $\text{Hb}(\text{OH})^{+}_{n-1}$. . . Hb^{++} . Hemoglobin behaves as though it were also a divalent base at various salt concentrations.

Since hemoglobin is an ampholyte dissociating both as acid and as base, the equilibrium must be considered simultaneously. The earlier equations for protein dissociation suggested that the neutral molecule at the isoelectric point was uncharged. In view of the extensive work on ampholytes stimulated by Bjerrum's (1) elaboration of the *Zwitter Ionen* hypothesis, it is probable that protein dissociation, also, can best be represented on this basis. The formula of the glycine *Zwitter Ion* is generally written $^+\text{NH}_3\text{CH}_2\text{COO}^-$. Comparably we may represent the neutral hemoglobin molecule as $^m\text{H}_n\text{Hb}^m-$ and since hemoglobin behaves as though it were divalent both as acid and as base, as $^{++}\text{H}_n\text{Hb}^-$.

When hemoglobin combines with alkali two hydrogen groups are supposedly given off, as in the classical theory, and there are $n-2$ left. The alkaline hemoglobin so formed contains two more

negative charges than positive charges and might be written $H_{n-2}Hb^-$. However, for reasons which will develop later it seems probable that the alkaline form can be better represented by the formula $^{++}H_{n-2}Hb^{--}$ in which the net charge is the same but the total charge is greater. The dissociation equation may be written



Combination with an acid, on the other hand, involves the addition of hydrogen ions. The acid hemoglobin may be written either $^{++}H_{n+2}Hb$ or $^{++}H_{n+2}Hb^-$. In both the net charge is the same, although the total charge varies. For our purposes we shall consider the species of lower charge, and write for its dissociation:



The above equations are to be considered, not as representing the actual changes in the molecule, but as a convenient schematic representation of the behavior of hemoglobin as a divalent acid and a divalent base.

The mass law equations written in terms of the activities of different species in the above reactions are, for dissociation on the acid side of the isoelectric point,

$$\frac{a_{H^+}^2 \times a_{^{++}H_nHb^-}}{a_{^{++}H_{n+2}Hb}} = K_1K_2 \quad (3)$$

where K_1 and K_2 are the constants for the dissociation of the first and second hydrogen atoms.

On the alkaline side we may write:

$$\frac{a_{H^+}^2 \times a_{^{++}H_{n-2}Hb^{--}}}{a_{^{++}H_nHb^-}} = K_3K_4 \quad (4)$$

K_3 and K_4 are the constants for the dissociation of the third and fourth hydrogen atoms. They have the same numerical values as would the classical acid dissociation constants whereas K_1 and K_2 are the hydrolysis constants of the classical basic dissociation constants.

In the presence of neutral salts stoichiometrical concentrations diverge widely from thermodynamic activities. The concentration of a saturated solution thus varies, whereas its activity remains constant. We must therefore write

$$a_{++H_nHb^-} = \gamma_{++H_nHb^-} [^{++H_nHb^-}] \quad (5)$$

where $a_{++H_nHb^-}$ is the activity, $\gamma_{++H_nHb^-}$ the activity coefficient, and $[^{++H_nHb^-}]$ the concentration. Similar expressions may be written for the other hemoglobin species. Substituting in the mass law expression yields the following equations.

$$\frac{a_{H^+}^2 [^{++H_nHb^-}]}{[^{++H_{n+2}Hb}]} = \frac{\gamma_{++H_{n+2}Hb}}{\gamma_{++H_nHb^-}} K_1 K_2 = K_1' K_2' \quad (6)$$

and

$$\frac{a_{H^+}^2 [^{++H_{n-2}Hb^{--}}]}{[^{++H_nHb^-}]} = \frac{\gamma_{++H_nHb^-}}{\gamma_{++H_{n-2}Hb^{--}}} K_3 K_4 = K_3' K_4' \quad (7)$$

The quantities experimentally determined and designated by the expression on the left hand side of equations (6) and (7) yield the products of the apparent dissociation constants, $K_1' K_2'$ and $K_3' K_4'$ respectively.

Assuming that we need take into account no other forms of hemoglobin, we may write:

$$S = [^{++H_{n-2}Hb^{--}}] + [^{++H_nHb^-}] + [^{++H_{n+2}Hb}] \quad (8)$$

where S is the solubility.

Let us substitute for each of the ionic species its value as defined in equations (6) and (7) in terms of their apparent dissociation constants and the hydrogen ion activity. The species in equilibrium with the saturating body $[^{++H_nHb^-}]$ is, for convenience, designated S_n .¹

$$\frac{S}{[^{++H_nHb^-}]} = \frac{S}{S_n} = 1 + \frac{a_{H^+}^2}{K_1' K_2'} + \frac{K_3' K_4'}{a_{H^+}^2} \quad (9)$$

¹ S_n as here employed is identical to the symbol S_n (3, 6) and β_n to β_0 (5) previously used in this laboratory.

If it be assumed that the dissociation constants are sufficiently far apart so that there is an inappreciable amount of $[^{++}\text{H}_n +_2\text{Hb}]$ in alkaline solutions, the term $\frac{a_{\text{H}^{+2}}}{K_1'K_2'}$ becomes negligible and we may write

$$\frac{S}{S_n} = 1 + \frac{K_3'K_4'}{a_{\text{H}^{+2}}} \quad (10)$$

and if there is an inappreciable amount of $[^{++}\text{H}_n -_2\text{Hb}^{--}]$ in acid solutions

$$\frac{S}{S_n} = 1 + \frac{a_{\text{H}^{+2}}}{K_1'K_2'} \quad (11)$$

The above relations suggest that the mass law, first applied to such systems where the concentration of the components was low and might as a first approximation be expected to yield the activities, holds also in any other electrolyte environment. In any constant environment, that is where $K_1'K_2'$ and $K_3'K_4'$ are constant, solubility should vary inversely as the square of the hydrogen ion activity where the protein dissociates as an acid, whereas where it dissociates as a base, solubility should vary as the square of the hydrogen ion activity.

The mode of representation of the charges on the hemoglobin molecule does not involve the adequacy of these equations for defining change in its solubility with pH. The justification for the assumption that hemoglobin acts as though it were divalent on either side of the isoelectric point rests upon the experiments. These may now be considered and the constants evaluated in terms of which solubility is described in various electrolyte concentrations.

Solubility of Hemoglobin in Concentrated Phosphate Solutions

Change of solubility with variation of hydrogen ion activity is most easily determined in heavily buffered solutions. We therefore first determined the solubility of hemoglobin in concentrated mixtures of K_2HPO_4 and KH_2PO_4 . The procedure was exactly the same as that described in the previous paper (10). Recrystallized horse carboxyhemoglobin was repeatedly equilibrated

TABLE I

Solubility of Carboxyhemoglobin at 25° in Concentrated Phosphate Solutions of Varying pH

Experiment No.	No. of determinations	Concentration phosphate	Mol fraction K_2HPO_4	Density	μ , ionic strength	pH	Solubility, Hb per liter	Solubility, Hb per 1000 gm. H_2O	β^*	pK'K'
Solubility in solutions more acid than pH 6.6										
		<i>mols per l.</i>					<i>gm.</i>	<i>gm.</i>		
10	2	1.683	0.274	1.1680	2.84	5.890	6.31	6.90	3.34	12.22
13	5	1.348	0.315		2.36	6.030	9.67	10.36	3.10	12.10
10	5	1.571	0.328	1.1613	2.82	6.058	3.95	4.28	3.11	12.18
10	4	1.686	0.330	1.1722	3.05	6.050	2.49	2.71	3.11	12.17
10	4	1.801	0.334	1.1840	3.28	6.054	1.45	1.65	3.10	12.15
10	4	1.908	0.338	1.1945	3.53	6.054	0.86	0.94	3.08	12.11
10	4	1.473	0.383	1.1553	2.80	6.224	3.28	3.53	3.01	12.30
10	4	1.367	0.452	1.1490	2.78	6.421	2.00	2.95	2.91	12.32
Average.										12.20
Solubility in solutions more alkaline than pH 6.6										
5	4	1.215	0.570	1.1406	2.75	6.70	2.77	2.93	2.89	13.98
4	5	1.553	0.594	1.1791	3.65	6.80	0.50	0.54	2.90	13.97
5	4	1.174	0.607	1.1375	2.74	6.80	3.21	3.38	2.94	13.97
5	4	1.092	0.690	1.1327	2.73	7.03	4.98	5.24	3.12	13.97
4	3	1.446	0.678	1.1717	3.63	7.00	0.86	0.92	3.16	13.84
4	3	1.357	0.696	1.1648	3.45	7.07	1.33	1.41	3.19	13.93
4	3	0.992	0.734	1.1227	2.56	7.16	10.54	11.09	3.29	13.95
4	4	1.268	0.735	1.1561	3.31	7.17	2.38	2.52	3.30	13.95
4	2	0.827	0.830	1.1125	2.34	7.43	38.6	41.1	3.67	14.02
5	7	0.981	0.825	1.1281	2.74	7.43	19.2	20.3	3.72	13.96
4	3	1.136	0.820	1.1467	3.16	7.43	8.40	8.85	3.73	13.95
4	3	1.298	0.810	1.1648	3.60	7.43	3.15	3.33	3.69	14.00
4	3	0.922	0.850	1.1201	2.65	7.53	29.6	31.5	3.82	14.05
Average.										13.96

* The average value for β at pH 6.6, as calculated in the preceding paper is 2.87.

with a given electrolyte solution at 25° until solubility remained constant over a number of days. Equilibration was facilitated by carrying out the final crystallization of the protein in solutions of the approximate salt concentration and pH used in the experiment. The pH of the buffer solutions was calculated by means of the equation of Cohn (4) derived from measurements upon more dilute phosphate solutions, and the saturated solutions checked electrometrically.

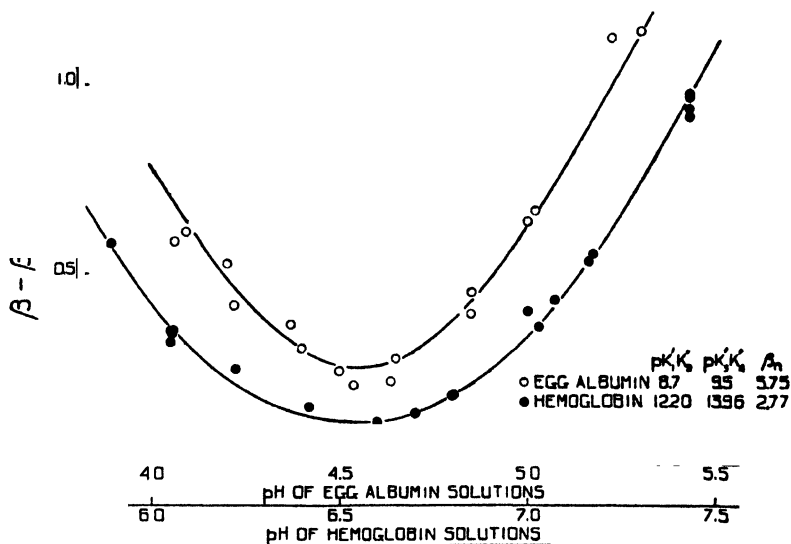


FIG. 1. The solubility of hemoglobin and of egg albumin in concentrated salt solutions of varying pH.

The concentration of protein in the successive filtrates was determined by nitrogen analyses and is recorded in Table I. The data for the solubility of carboxyhemoglobin in concentrated phosphate solutions at pH 6.6 and 25° were given in Paper VIII of this series (10). Densities were determined on hemoglobin phosphate solutions, and the concentration of phosphate assumed to be that of the buffer solutions added to the protein.

The equation previously employed, defining solubility of hemoglobin as well as of other proteins in concentrated salt solutions, is

$$\log S = \beta - K_s' \mu \quad (12)$$

in which S is the solubility at the ionic strength, μ (17); and β the value of $\log S$ when $\mu = 0$. Since, however, hemoglobin is dissolved by low concentrations of salt as well as being precipitated by higher concentrations, the constant β has only hypothetical significance. K_s' is the apparent salting out constant whose numerical value varies with the nature of the protein and salt. For hemoglobin in phosphate solutions it is independent of temperature and pH and equal to 0.88.

Since K_s' is independent of pH, the variation of solubility with reaction is completely defined by the variation of β . Values of β calculated by equation (12) are given in Table I. They are graphically represented in Fig. 1 in such a manner that their variation with pH may be compared with the remarkably similar curve for the solubility of egg albumin in concentrated ammonium sulfate solutions derived by Cohn (3) from the data of Sørensen and Høyrup (27). We have calculated also the values of β for egg albumin on the acid side of the pH of minimum solubility by equation (12). (In these calculations K_s' for egg albumin has been taken as 0.92.) The pH of minimum solubility and the values of β are of course quite different for the two proteins. The adequacy of the equation for the characterization of solubility in concentrated salt solutions of varying pH is demonstrated to be as satisfactory for hemoglobin as for egg albumin.

The relation between solubility and salt concentration, defined in equation (12), is logarithmic in form since it is the logarithm of the activity coefficient which varies with the free energy. The mass law equations in terms of which dissociation is described have a different origin. Moreover, the forms of equations (10) and (11) preclude their transformation into a simple logarithmic expression. In the estimation of dissociation constants we must therefore substitute not values of β , the logarithm of hypothetical solubilities in the absence of salt, at different values of pH, but their antilogs. These, when substituted for S in the above equations, should permit estimation of the dissociation constants. For $\frac{S}{S_n}$

we may substitute $\frac{\text{antilog } \beta}{\text{antilog } \beta_n}$, where β_n is defined by this relation:

$$\frac{S}{[+H_nHb^-]} = \frac{S}{S_n} = \frac{\text{antilog } \beta}{\text{antilog } \beta_n} \quad (18)$$

The constancy of K_s' at varying pH values is our justification for this simple treatment.

On this basis the values of the antilog of β when plotted against the reciprocal of the square of the hydrogen ion activity on the alkaline side of pH 6.6 should fall upon a straight line. The intercept is, according to equation (10), the solubility of the equally charged molecule $[^{++}H_nHb^-]$ designated S_n , and the slope is the product of the solubility of this molecule and its dissociation constants.

Analysis of the experimental material in terms of equation (10) by means of simultaneous equations calculating each point against all the other points yields for the antilog of β_n the value 0.61×10^3 and, for $K_s'K_4'$, 1.03×10^{-14} (5).

On the acid side of the solubility minimum the evaluation of the dissociation constants depends on the linear relation between the solubility or the antilog of β and the square of the hydrogen ion activity. Calculations for $K_1'K_2'$ analogous to those for $K_s'K_4'$, on the basis of equation (11), yield, for the antilog of β_n , 0.69×10^3 and, for $K_1'K_2'$, 0.82×10^{-12} .

The dissociation constants calculated above demonstrate that there is an overlapping of the ranges of alkaline and acid dissociation. This results in higher estimates of S_n or β_n on the basis of the simplified expressions (equations (10) and (11)) than would result from the more complete expression (equation (9)), in which, besides the species in equilibrium with the saturating body, more positively and more negatively charged forms are simultaneously considered.

As a first approximation let us use the values derived above and solve equation (9) for pH 6.6, substituting for the ratios of the dissociation constants and hydrogen ion activity; then

$$\frac{S}{S_n} = \frac{\text{antilog } \beta}{\text{antilog } \beta_n} \quad 1 + 0.163 + 0.061 = 1.224$$

instead of 1.163 as determined from the alkaline side alone or 1.061 as determined from the acid side alone. The corrected values for antilog β become

$$\frac{1.163}{1.224} \times 0.61 \times 10^3 = 0.58 \times 10^3$$

or

$$\frac{1.061}{1.224} \times 0.69 \times 10^3 = 0.60 \times 10^3$$

The average of the above values, 0.59×10^3 , may be taken and β_n is therefore 2.77.

The values of the dissociation constants may now be corrected by substituting in equation (9) the corrected value of S_n or anti-log β_n and of the approximate value of $pK_1'K_2'$ when evaluating $pK_3'K_4'$ and of $pK_3'K_4'$ when calculating $pK_1'K_2'$. These corrected values are given in the last column of Table I. The negative logarithm of the average values of the dissociation constants is also given. Thus $pK_1'K_2'$ becomes 12.20 rather than 12.08 as calculated above and $pK_3'K_4'$, 13.96 rather than 13.99. On this basis $K_1'K_2'$ is 0.63×10^{-12} and $K_3'K_4'$ is 1.10×10^{-14} . These values have been employed in plotting the values of $\beta - \beta_n$ against pH in Fig. 1.

Values for the dissociation constants for egg albumin in ammonium sulfate have been calculated similarly, as a result of which, β_n becomes 5.75, $pK_1'K_2'$, 8.7, and $pK_3'K_4'$, 9.5. The curve in Fig. 1 has been calculated from these constants.

The linear relationship required by equations (10) and (11) is shown graphically in Fig. 2. In the range where there is appreciable overlapping of the constants the straight lines have been dotted and the solid lines are continued according to equation (9). In the same figure are shown some of the data for solubility in dilute salt solutions taken up in the next section.

Solubility of Carboxyhemoglobin in Relatively Dilute Phosphate Solutions

The change of solubility of hemoglobin with changing hydrogen ion activity is less easily determined the less concentrated the buffer solution, for the pH of the saturated solution is not always that of the original buffer solution. Hemoglobin is itself a competent buffer. As a result it is more difficult to attain a given pH, and both pH and solubility must be measured.

Equilibrium with the salt solution also is less easily reached the more dilute the electrolyte. The hemoglobin was therefore prepared in such a manner that the salts present in the solution from which the protein was crystallized were potassium phosphates.

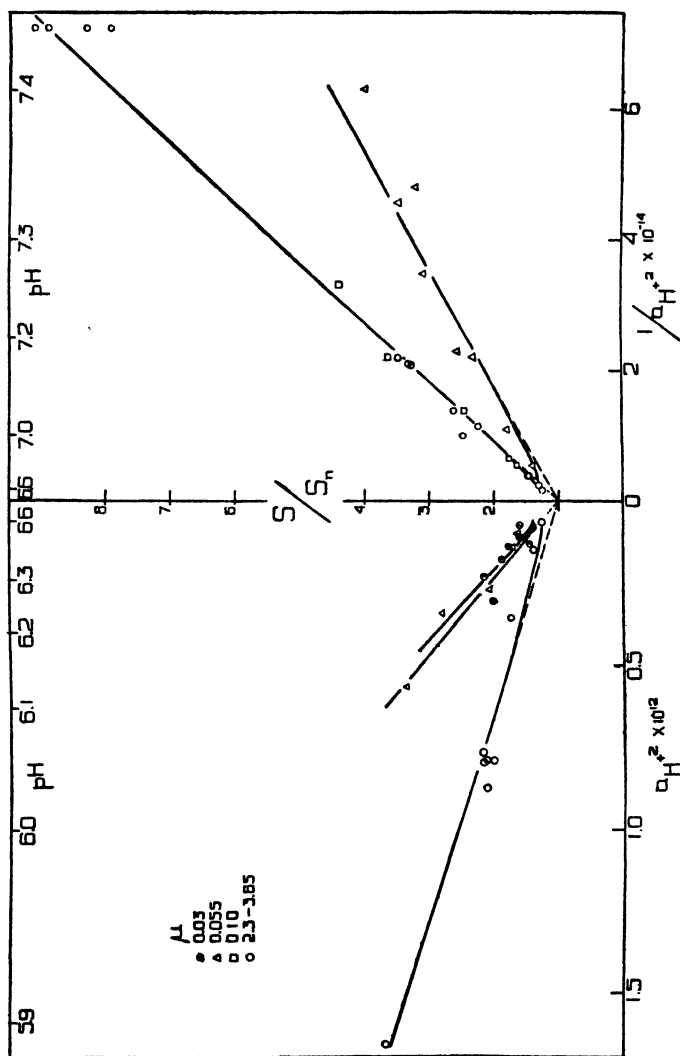


FIG. 2. The solubility of hemoglobin in phosphate buffers of constant ionic strength and varying pH

Also, in the experiments in which the phosphate concentration was very low, 0.02 M and below, the concentration was checked by colorimetric phosphate determinations by the method of Fiske and Subbarow (9). In concentrations of salt less than 0.412 M the mol fraction of K_2HPO_4 was calculated from the phosphate equation (reference (4)) at the pH experimentally determined by the hydrogen electrode.

The experimental results are given in Table II. For the most part density determinations were found to be unnecessary. The composition of solutions of hemoglobin and electrolytes of varying concentration has been analyzed and demonstrates that the specific volume of hemoglobin is approximately 0.71. Accordingly the ionic strength has been calculated on this basis as was also the solubility per 1000 gm. of water.

The theoretical interpretation of the solubility of hemoglobin in dilute as well as in concentrated salt solutions involves the consideration of both electrolyte concentration and hydrogen ion activity. In the previous section it was demonstrated that at a constant electrolyte environment, solubility varied with pH as though hemoglobin were a divalent acid or base.

The experiments that are reported demonstrate that this principle applies also in dilute phosphate solutions. If at any given ionic strength the solubility of the hemoglobin be plotted against $\frac{1}{a_{H^+}}$ on the alkaline side of pH 6.6, or against $a_{H^+}^2$ at reactions acid to the same pH, the experimental points fall upon straight lines. Solubility of hemoglobin at different hydrogen ion activities is compared with that at pH 6.6, since this approximately coincides with the minimum solubility, certainly for concentrated solutions. The difference between the solubility at the given pH and at pH 6.6 ($S - S_{6.6}$) has been calculated and is recorded in Table II. On the basis of the relation given by equations (10) and (11), and employed in concentrated phosphate solutions, the solubility of the neutral molecule, S_n , and also the apparent dissociation constants, $K_1'K_2'$ and $K_3'K_4'$, have been calculated and are recorded in the eleventh column of Table II.

The assumptions implied in the use of these equations are that the concentration of more negatively charged ions is negligible at reactions acid to pH 6.6 and the concentration of more positively

TABLE II

Solubility of Carboxyhemoglobin in Dilute Phosphate Solutions of Varying Hydrogen Ion Activity at 25°

Experiment No.	No. of determinations	Concentration phosphate	Mol fraction KH_2PO_4	μ , ionic strength	pH	Solubility, Hb per liter	Solubility, Hb per 100 gm H_2O	$S - S_{\text{H}_2\text{O}}$	S_{H}	pK'K'	S_{H} corrected	pK'K' corrected
Solubility at pH 6.6												
		<i>mols per l.</i>				<i>gm.</i>	<i>gm.</i>					
9	4	0.01	0.28	0.016	6.59	26.4	26.9					
9	1	0.01	0.30	0.016	6.64	24.9	25.3					
8	1	0.02	0.29	0.032	6.57	26.7	27.3					
30	3	0.02	0.31	0.033	6.60	26.4	27.0					
8	2	0.02	0.33	0.033	6.62	26.4	26.9					
3	4	0.024	0.32	0.040	6.60	30.0	30.6					
30	8	0.04	0.35	0.070	6.60	31.5	32.3					
3	3	0.07	0.379	0.122	6.60	31.0	31.8					
31	7	0.100	0.405	0.186	6.60	36.5	37.5					
2,3	5	0.135	0.426	0.259	6.60	38.1	39.5					
12,5	10	0.325	0.485	0.671	6.60	52.2	52.8					
5	6	0.498	0.505	1.050	6.60	35.0	36.7					
5	4	0.688	0.517	1.46	6.60	22.2	23.2					
3	4	0.879	0.524	1.88	6.60	11.2	11.7					
4	4	0.879	0.524	1.88	6.60	14.1	14.7					
Solubility in solutions more acid than pH 6.6												
9	1	0.01	0.2	0.014	6.41	29.3	29.8	5.8	19.8	12.55		12.63
9	3	0.01	0.24	0.015	6.49	28.3	29.1	4.8	17.1	12.79		12.74
Average.....									18.5	12.67	17.9	12.68
8	1	0.02	0.16	0.026	6.26	38.9	39.9	14.2	21.9	12.46		12.52
13	4	0.02	0.19	0.028	6.32	42.1	43.4	17.4	19.0	12.71		12.71
8	2	0.02	0.21	0.028	6.38	36.1	37.0	11.0	19.7	12.64		12.68
18	1	0.02	0.21	0.028	6.385	34.2	34.9	8.9	20.7	12.61		12.64
18	1	0.02	0.24	0.030	6.44	27.8	28.4	2.2	24.2	(12.19)		12.49
8	1	0.02	0.24	0.030	6.45	31.3	32.0	5.8	20.4	12.65		12.67
8	3	0.02	0.26	0.031	6.49	31.2	31.8	5.6	17.7	12.82		12.73
Average.....									20.5	12.67	19.8	12.64
30	1	0.04	0.294	0.065	6.50	34.5	35.4	5.6	20.3	12.82		12.78
30	1	0.04	0.26	0.062	6.43	36.1	37.1	7.5	23.3	12.64		12.69
18	3	0.04	0.20	0.059	6.29	42.3	43.9	14.5	24.8	12.50		12.58
18	2	0.04	0.18	0.055	6.24	58.2	60.7	31.7	21.5	12.72		12.74
18	1	0.04	0.12	0.050	6.12	70.0	73.0	44.5	23.5	12.58		12.62
Average.....									22.7	12.67	21.7	12.69

TABLE II—*Concluded*

Experiment No.	No. of determinations	Concentration phosphate	Mol fraction K_2HPO_4	μ , ionic strength	pH	Solubility, Hb per liter	Solubility, Hb per 1000 gm. H_2O	$S - S_{\infty}$	S_n	pK'/K'	S_n corrected	pK'/K' corrected
Solubility in solutions more acid than pH 6.6— <i>Concluded</i>												
		mols per l.				gm.	gm.					
13	4	0.114	0.16	0.160	6.05	75.0	79.5	43.5	32.2	12.27	29.6	12.33
13	4	0.220	0.20	0.330	6.07	76.1	81.2	37.4	40.2	12.15	36.3	12.24
13	4	0.412	0.23	0.645	6.03	73.8	79.3	27.3	49.9	11.83	44.5	11.95
13	5	0.895	0.282	1.490	6.03	40.2	42.9	18.9	22.5	12.02	20.3	12.10
Solubility in solutions more alkaline than pH 6.6												
8	4	0.005	0.3	0.008	6.72	21.1	21.3					
9	2	0.01	0.36	0.017	6.74	23.4	23.8					
8	3	0.02	0.36	0.035	6.69	24.5	25.0					
30	1	0.02	0.46	0.039	6.87	29.1	29.8	2.6	26.1	14.60		14.25
30	1	0.02	0.55	0.044	7.02	37.6	38.5	10.7	26.0	14.37		14.17
30	1	0.02	0.64	0.047	7.17	47.6	49.4	21.4	26.3	14.40		14.23
18	2	0.02	0.65	0.048	7.18	52.1	54.3	26.0	26.4	14.34		14.18
30	1	0.02	0.69	0.050	7.27	62.7	65.7	37.2	26.7	14.38		14.23
13	2	0.02	0.73	0.052	7.33	70.3	74.3	45.7	27.0	14.41		14.27
18	1	0.02	0.73	0.052	7.34	65.2	68.4	39.8	27.3	14.50		14.34
30	1	0.02	0.76	0.052	7.40	80.6	85.8	56.8	27.5	14.46		14.33
Average.....									26.7	14.43	21.4	14.25
30	1	0.04	0.50	0.082	6.87	39.0	40.2	8.9	27.5	14.10		13.96
30	1	0.04	0.53	0.089	6.91	41.5	43.0	11.3	27.9	14.09		13.96
30	1	0.04	0.62	0.094	7.07	57.0	59.5	27.5	28.2	14.10		13.99
30	1	0.04	0.675	0.100	7.17	83.0	88.4	55.9	27.8	14.01		13.92
12	1	0.039	0.66	0.095	7.16	75.0	79.7	47.7	27.8	14.05		13.96
13	2	0.039	0.715	0.103	7.26	98.2	106.6	73.9	28.8	14.08		13.99
Average.....									28.0	14.07	24.3	13.96
12	2	0.114	0.75	0.314	7.22	109.5	119.5	76.3	38.2	14.12		14.07
13	1	0.114	0.79	0.332	7.31	134.0	150.0	106.2	39.3	14.17		14.12
Average.....									38.8	14.14	35.9	14.10
12	7	0.520	0.843	1.560	7.40	104.7	116.5	94.8	19.1	14.09	18.2	14.07

charged ions is negligible at reactions alkaline to pH 6.6. However, these assumptions are no more justified in dilute than in concentrated solutions. The estimates of the solubility of the equally charged molecule, S_n , and of the dissociation constants are therefore subject to correction for the overlapping of the latter. The values of the quantities $\left(\frac{a_{H^+}{}^2}{K_1'K_2'}\right)$ and $\left(\frac{K_3'K_4'}{a_{H^+}{}^2}\right)$ at pH 6.6 have been estimated at the various ionic strengths on the basis of the

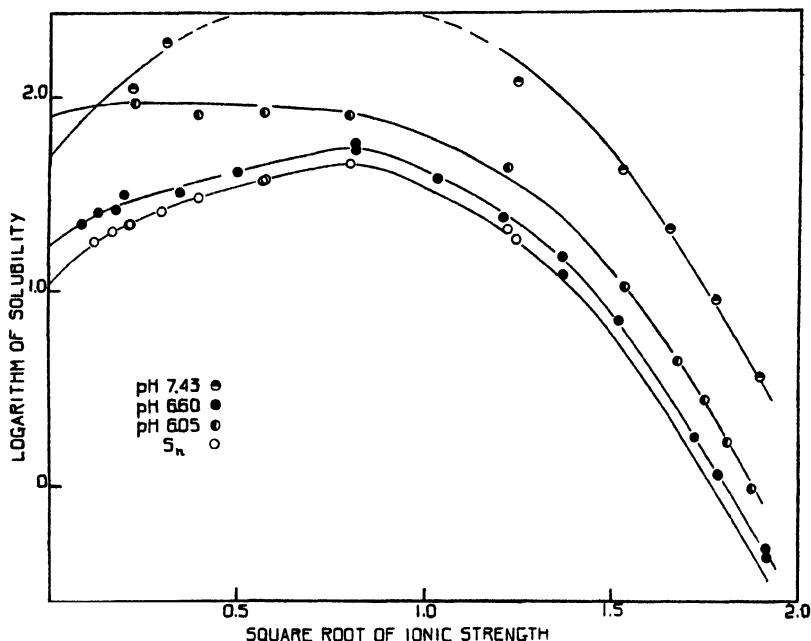


FIG. 3. The solubility of hemoglobin at constant pH

dissociation constants given in Table II (eleventh column). These, substituted in equation (9) by the same procedure as was used in concentrated salt solutions, yield values of S_n corrected for the presence of both positively and negatively charged molecules. These estimates of S_n are graphically represented in the accompanying Fig. 3, where they may be compared with the actual solubility measurements carried out at pH 6.6, and also at other reactions, pH 6.05 and 7.43, at which a number of measurements have also been made.

The dissociation constants are recalculated according to equation (9) with the corrected values for S_n and the approximate value for $K_1'K_2'$ when calculating $K_3'K_4'$ and *vice versa*. Their negative logarithms and the negative logarithms of their averages are recorded in the thirteenth column. Each ionic strength is treated separately in the more dilute phosphate solutions because of the more complex nature of the relation between ionic strength and the activity coefficient that prevails when the salting out constant does not dominate the latter.

The linear relation between $\frac{S}{S_n}$ and a_{H^+2} or its reciprocal is graphically demonstrated in Fig. 2, where values of $\frac{S}{S_n}$ fall upon the same straight line at constant ionic strength. At reactions near the isoelectric point, due to the overlapping of the constants, the experimental points do not fall upon the straight line and the curve describing the deviation was drawn according to equation (9) with use of the corrected values of $K'K'$, the dissociation constants, whereas the dotted lines describe the relationships in equations (10) and (11).

The apparent dissociation constants vary with electrolyte concentration. The values of $pK_1'K_2'$ and also of $pK_3'K_4'$ appear to be constant in concentrated salt solutions as we saw in the first section of this paper, but they become greater in the more dilute concentrations of phosphate. With decrease in concentration of salt $pK_1'K_2'$ moves toward pH 6.6 and $pK_3'K_4'$ moves away from it. If one plots the values of the apparent acid constants and extrapolates to find the value in the absence of salt, pK_1K_2 is found to be approximately 12.90. The variation of $pK_3'K_4'$ is in the same direction, but not so well defined. pK_3K_4 is, however, approximately 14.3. The magnitude of the variation of the acid and basic constants with salt concentration would be more concordant if slightly lower values of S_n were employed in the range of maximum solubility.

If one could evaluate the activity coefficient of the neutral molecule and the variation of $pK'K'$, one could calculate the activity coefficients at any pH. In concentrated solutions the activity coefficients of the neutral molecule have been defined by the relation: $\log S_n = 2.77 - 0.88\mu$ and the ratio $\frac{S_{6.6}}{S_n}$ is 1.26. This

ratio depends upon the value of the dissociation constants and the degree of their overlapping. It is, however, approximately constant in all concentrations of salt, although somewhat larger when the concentration of salt is very low.

The increase in solubility of S_n with salt concentration in dilute solutions up to $\sqrt{\mu} = 0.5$ may be approximately described by means of the simplified Debye-Hückel equation used to describe the solubility of oxyhemoglobin in phosphate buffers at pH 6.6 and 0° by Cohn and Prentiss (7).

$$-\log \gamma = \log S - \log S_0 = \frac{2 \sqrt{\mu}}{(1 + 1.5 \sqrt{\mu})} - K_s \mu \quad (14)$$

where S is the solubility and S_0 the solubility in the absence of salt. The salting out constant K_s , though not employed by Cohn and Prentiss is necessary here.

If the solubility of the neutral molecule in the absence of salt be assumed to be 11 gm. per liter, the smooth curve drawn through the calculated values of S_n up to $\sqrt{\mu} = 0.5$ in Fig. 3 may be described by the above equation if $\log S_0$ of the neutral molecule be 1.04 and $K_s 0.3$.

This equation, used here for purposes of interpolation, would signify, if referring to a salt, that it was either bi-bivalent or uni-quadrivalent. In considering such a highly charged molecule as hemoglobin it was pointed out by Cohn that: "To conclude that oxyhemoglobin is bivalent or quadrivalent might be correct, but would be unjustified. . . . Oxyhemoglobin behaves. . . . as though it were bivalent or quadrivalent" ((8) p. 637). Furthermore, the applicability to protein solutions of equations specifically derived for *Zwitter Ionen* will subsequently appear from this laboratory.

In developing the expressions for the dissociation of hemoglobin, the mode of representation was arbitrarily chosen such that the predominantly alkaline species had a higher number of charges than the neutrally charged molecules and the predominantly acid forms a still lower number. If, knowing the variation of pK_3'/K_4' and pK_1'/K_2' with salt concentration and the values of S_n , one calculates the activity coefficients of the more negatively charged molecule, the results suggest that the apparent valence type is

greater than that of the neutral molecule, since salt has here a larger solvent action. The acid species is of a lower apparent valence type, and is far more easily "salted out." This relative increase in apparent charge in more alkaline solutions is independent of the form of equation in terms of which solubility is described, and is even more marked in casein than in hemoglobin.

The variation of $pK_1'K_2'$ and $pK_3'K_4'$ with salt concentration involves a variation of the apparent isoelectric point $\frac{1}{2}(pK_1'K_2' + pK_3'K_4')$ with salt concentration, from a higher to a lower value. This results from variation of the pH of minimum solubility. The sum of the terms $\left(\frac{a_{H^+}{}^2}{K_1'K_2'}\right)$ and $\left(\frac{K_3'K_4'}{a_{H^+}{}^2}\right)$ has a minimum value when they are equal to each other and $a_{H^+}{}^4 = K_1'K_2'K_3'K_4'$ (20). If the validity of the dissociation constants deduced from solubility data be accepted, and these be employed in the calculation of the isoelectric point, change in the isoelectric point with change in salt concentration results. Whether the isoelectric point as defined by cataphoretic measurements also changes with salt concentration is more difficult to demonstrate conclusively, so fraught with technical difficulties are mobility measurements in the solutions of different conductivity. This change in the pH of minimum solubility with salt concentration and also with the character of the salt was first noted by Hardy (11) in experiments upon serum globulin.

The solubility of hemoglobin, then, in various concentrations of salt and at various reactions may be described as an equilibrium between the neutral molecule, either uncharged as on the classical hypothesis, or equally charged as a *Zwitter Ion*, and a more negatively charged species as well as a more positively charged species. Their quantitative relationships are described by equation (9). All of the forms are present at any pH in a greater or a lesser amount. The concentration obviously depends upon the relation between $pK'K'$ and pH and also between the $pK'K'$ values for acid and basic dissociation. The nearer the numerical values of the dissociation constants to each other, the greater the overlapping and the greater the relative concentration of acid and alkaline forms present at any pH. Even at the pH of minimum solubility hemoglobin contains approximately 20 per cent of the positive and negative species. Egg albumin in concentrated am-

monium sulfate solutions contains even more unequally charged molecules as may be seen in Fig. 1 since $\beta - \beta_n$ and therefore $\frac{S}{S_n}$ is greater for egg albumin than for hemoglobin. This follows from the dissociation constants being much closer together. Analysis of their titration curves, by Cohn (3), also suggested that the dissociation constants of egg albumin overlap to a greater extent than do those of hemoglobin. The solubility of the neutral molecule of casein is so small as to be almost immeasurable, but the activity coefficients of the unequally charged molecules and the variation of the apparent isoelectric point can also be calculated, as we shall see in the next section, on the basis of equation (9) in which the solubility at various reactions is adequately described if the protein be assumed to behave as though it were a divalent acid and base.

Solubility of Casein in Systems Containing NaCl and NaOH or HCl

Within the past few years there have been published several investigations upon the solubility of casein in salt solutions at varying hydrogen ion activities (18, 19, 29). These have for the most part come from the Carlsberg laboratory and have been the basis of a controversy between Sørensen and Ostwald in the *Kolloid-Zeitschrift*.

It is not our purpose in this communication to discuss every aspect of the experiments upon casein that they considered. Problems which may involve complicating factors because casein, unlike hemoglobin, is not a chemical individual, are not part of our present concern. As the description developed by Cohn (6) from his study of the solubility of casein in dilute solutions of sodium hydroxide has now been extended to solutions of other proteins in different electrolyte environments, it seemed appropriate to determine whether this relation does not also apply to the measurements of Sørensen and Sládek and of Linderstrøm-Lang and Kodama upon the solubility of casein.

Sørensen and Sládek (29) studied the solubility of casein in NaOH in the presence of NaCl. Since they found that solubility was dependent upon the concentration of the saturating body they preferred to express results in terms of the "per cent dissolved." In order to consider only that aspect of the problem that is com-

parable to hemoglobin investigations, in which solubility is independent of the amount of saturating body, we have recalculated data in which a constant amount of saturating body was employed, namely about 8.5 gm. of casein per liter. The results, expressed in terms of milligram equivalents of nitrogen per liter, have been transformed into gm. of protein per liter, it being assumed that casein contains 15.6 per cent nitrogen.

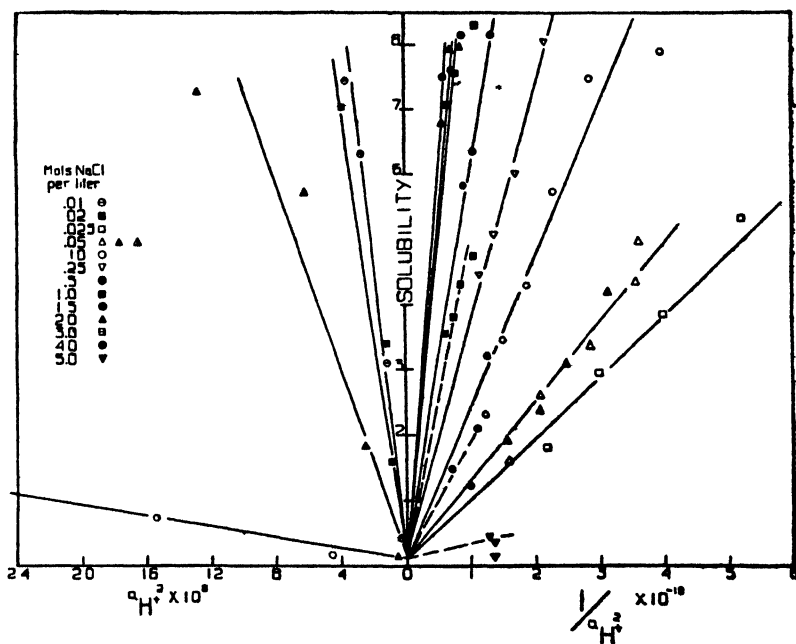


FIG. 4. The solubility of casein at varying pH from the experiments of Linderstrøm-Lang and Kodama and of Sørensen and Sládek.

In Fig. 4 are plotted some of Sørensen's results, from his Tables 4, 6, and 7, the solubility, in gm. per liter, being plotted against $\frac{1}{a_{H^2}}$ at constant salt concentration. It may be seen that the points representing the solubility of the casein-sodium hydroxide-sodium chloride-containing systems fall upon straight lines. Casein thus acts as a divalent acid in the presence of electrolytes as in their absence (6). It is to be noted that the slopes of the

lines vary, revealing apparent rather than true dissociation constants.

The solubility of casein in HCl in the presence of NaCl was determined by Linderstrøm-Lang and Kodama (19) by the same technique as was subsequently employed by Sørensen. They found also that solubility depended upon the amount of saturating body present. In Fig. 4 we have also plotted against a_{H^+} ² those of their results in which the total protein present was approximately the same as in the NaOH-containing systems considered; actually, 8.2 gm. per liter.

On the acid side of its isoelectric point casein also behaves as though it were divalent. As in hemoglobin the slope constants vary in the opposite direction from those on the alkaline side. Salt in all concentrations diminishes solubility in the presence of free acid, an observation stressed by Sørensen regarding these data and by Osborne (22) and others regarding various other globulins.

The measurements upon casein in NaCl solutions at different hydrogen ion activities may be employed precisely as have those upon hemoglobin in calculating apparent dissociation constants by means of equation (9). The results of these calculations are reported in Table III. They show not only the diverse nature of the influence of salt on the predominantly positively or negatively charged casein molecules, but also the change in the apparent isoelectric point, $\frac{1}{4} (pK_1'K_2' + pK_3'K_4')$, from a value consistent with that generally recorded in the literature to one at a relatively acid reaction. That the point of maximum precipitation of casein varied with the salts present was shown by Michaelis and von Szent-Györgyi (21).

The quantities in Table III reveal the change in the activity coefficients of the variously charged casein molecule with change in salt concentration.

$$S_n K_3' K_4' = S_n \left(\frac{\gamma_{S_n}}{\gamma_{P^-}} K_3 K_4 \right) = \frac{S_{n_0}}{\gamma_{S_n}} \left(\frac{\gamma_{S_n}}{\gamma_{P^-}} K_3 K_4 \right) = \frac{S_{n_0} K_3 K_4}{\gamma_{P^-}} \quad (15)$$

and

$$pK_3' K_4' - \log S_n = pK_3 K_4 - \log S_{n_0} + \log \gamma_{P^-} \quad (16)$$

where P^- is the predominantly negatively charged molecule, S_n is the solubility of the neutral molecule in the presence of, and S_{n_0} the solubility in the absence of, salt.

$$\frac{S_n}{K_1'K_2'} = \frac{S_n}{\gamma_{P^{++}} K_1K_2} \frac{S_{n_0} \gamma_{S_n}}{\gamma_{S_n} \gamma_{P^{++}} K_1K_2} \frac{S_{n_0}}{K_1K_2 \gamma_{P^{++}}} \quad (17)$$

$$\log S_n + pK_1'K_2' = \log S_{n_0} + pK_1K_2 - \log \gamma_{P^{++}} \quad (18)$$

where P^{++} is the predominantly positively charged molecule. The variation of the activity coefficient of the form of casein that is

TABLE III
Activity Coefficients of Casein*

μ	0	0.01	0.02	0.025	0.05	0.1	0.25	0.5	1.0	1.5	2.0	3.0	4.0	5.0
$pK_1'K_2' - \log S_n$	10.45			10.05	9.90	9.65	9.45	9.25	9.0	8.9	9.0	9.35	9.74	10.60
$pK_1'K_2' + \log S_n$	8.70	8.4	8.25		7.85	6.6								
$pK_1'K_2' - pK_1K_2$	4.8		4.59	4.44	4.06									
4														

* Calculated from the data of Linderström-Lang and Kodama (19) and of Sørensen and Sládek (29), in which the total casein present is 8.2 to 8.5 gm. per liter.

present in acid solution is apparently dominated by a salting out constant, since the results are adequately described by the equation

$$-\log \gamma_{P^{++}} = -19 \mu$$

The activity coefficients in alkaline solution pass through a minimum as do those of most ions and many proteins. The values of $pK_1'K_2' - \log S_n$ given in Table III may be plotted against $\sqrt{\mu}$. The intercept is $pK_1K_2 - \log S_{n_0}$ and has the extrapolated value 10.45. The curve describes the values of $\log \gamma_{P^-}$ according to equation (16). If we employ the Debye-Hückel equation for interpolation in the same manner in which it has been used for hemoglobin we find that $-\log \gamma_{P^-} = \frac{3\sqrt{\mu}}{(1+0.7\sqrt{\mu})} - 0.4\mu$. This relation describes the data satisfactorily except in the most

concentrated solutions. Again, however, as in hemoglobin, we must not consider this equation as final since the application to proteins of equations describing the electrical interaction of *Zwitter Ionen* with electrolytes and non-electrolytes will later appear from this laboratory.

Regardless of the form of the equation, in casein also we have a molecule of apparently higher charge formed by the combination with alkali and a molecule of lower charge formed upon the addition of acid. That is, if increase in solubility in dilute concentrations of salt is due to the solvent action of the electrolyte and is dependent upon the apparent valence type of the molecule the predominantly negatively charged molecule is much more highly charged than the predominantly positively charged molecule which is salted out.

In conclusion, the change in solubility of casein, as well as of hemoglobin and egg albumin, in the presence of acid or alkali and in salts of varying concentration can be described if the neutral, or isoelectric, molecule be assumed to be in equilibrium with more positively and more negatively charged molecules; and if, further, their quantitative relationship is expressed by mass law expressions in which the protein behaves as though it were a divalent base and a divalent acid.

I wish to thank Professor Edwin J. Cohn for his kind advice and interest during the course of this investigation and for suggesting the application of the mass law in describing solubility in the presence of salts, as reported by Cohn and Green (5).

SUMMARY

1. The solubility of carboxyhemoglobin in potassium phosphate buffers of varying concentration and pH at 25° is described.
2. Hemoglobin behaves as though it were a divalent acid or base on either side of the pH of minimum solubility.
3. The total solubility, S , is related to the solubility of the neutral molecule, S_n , according to the equation

$$\frac{S}{S_n} = 1 + \frac{K_2'K_4'}{a_{H^+}^2} + \frac{a_{H^+}^2}{K_1'K_3'}$$

where K_3/K_4' and K_1/K_2' are the apparent dissociation constants of the protein at constant ionic strength.

4. The apparent dissociation constants and isoelectric point vary with the electrolyte concentration. pK_1/K_2' and pK_3/K_4' increase in value as the salt concentration increases.

5. The solubility of casein in NaCl and either NaOH or HCl may be described if casein also is assumed to behave as though it were a divalent acid or base.

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HYPOCALCEMIA FOLLOWING EXPERIMENTAL HYPER-PARATHYROIDISM AND ITS POSSIBLE SIGNIFICANCE

BY AARON BODANSKY AND HENRY L. JAFFE

(From the Laboratory Division, Hospital for Joint Diseases, New York)

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We have shown that in the guinea pig hypercalcemia, with and without hyperphosphatemia, may be produced by suitably large single doses of parathormone, and more easily in young than in adult animals. When repeated doses were given to young guinea pigs for 34 days up to within 24 hours of the serum analysis, hypercalcemia without hyperphosphatemia was in evidence (1).

We observed subsequently that hypocalcemia appeared with seeming regularity after prolonged parathormone treatment in guinea pigs which were receiving their regular diet and were therefore on an adequate calcium intake. The hypocalcemia did not appear during the course of the administration of parathormone but several days after its discontinuance.

Five young guinea pigs received during a period of at least 4 days doses which were increased from 30 to 60 units daily. This treatment had been preceded by periods of varying length (2 to 3 weeks) on smaller doses. Serum calcium and phosphorus were determined 4 and 5 days after the last dose. Hypocalcemia and hyperphosphatemia were regularly shown. One guinea pig, similarly treated but tested 7 days after the last dose, showed normal serum calcium and phosphorus (Table I). Litter mate controls showed normal calcium and phosphorus values.¹

¹ In apparently normal guinea pigs, young and adult, the serum calcium was found to be within the range 10.5 ± 0.5 mg. per 100 cc., values outside of this range ("low normal" and "high normal") being rare. (Subsequent findings were in line with the earlier results.) We therefore felt justified in attaching significance not only to frankly hypercalcemic values but also to a high proportion of high normal values found in experimental hyperparathyroidism (1). Similarly hypocalcemia may be manifested not only

These results were quite different from those obtained at shorter intervals after the last injection (1). The following interpretation of these results was possible: Parathormone administration reduces the calcium reserves of the tissues (ultimately and principally the bone); upon the discontinuance of parathormone injections, calcium is reabsorbed into the bone at such rate as to cause hypocalcemia. The histological evidence of bone decalcification and subsequent rapid repair supported this interpretation (2).

Our observation presented an interesting analogy to the observation of hypocalcemia (and frequently tetany) after the removal of a parathyroid adenoma in clinical hyperparathyroidism (3-6,

TABLE I
Effect of Parathormone Administration

Guinea pig No.	Weight at end of experiment	Days after last injection	Serum Ca	Serum P
	gm.		mg per 100 cc.	mg. per 100 cc.
870	480	4	9.4	7.7
905	400	4	8.0	8.0
907	480	4	7.4	7.8
901	350	5	7.6	8.4
903	370	5	8.8	4.8
906	370	7	10.2	6.9

and others). (The removal of one normal parathyroid, as is well known, causes no apparent disturbance of calcium and phosphorus metabolism.)

On the basis of clinical data in one such case, Snapper suggested the following explanations of the development of hypocalcemia.

"The possibility of an injury to the other parathyroids during the operation is obvious. In the present case, however, the right side of the thyroid was not exposed. The parathyroids situated at the right side could not be injured, therefore, during the operation. The possibility also exists that this patient had no parathyroids on the right side, and that immediately after the operation the only remaining left parathyroid gland

by very low calcium values, but also by a high proportion of low normal values.

Serum inorganic phosphorus values vary with age from about 8.0 mg. per 100 cc. for guinea pigs weighing less than 300 gm. to about 3.0 mg. for adult guinea pigs (1).

was not able to regulate the calcium metabolism adequately. Meanwhile, if one does not wish to use the unproved hypothesis that the patient had no parathyroids on the right side, another possibility may be considered. After the operation the decalcified skeleton may absorb calcium from the blood with so much avidity that through this rapid absorption the calcium content of the serum descends below the critical level, and symptoms of tetany appear; even three normal parathyroids would then be unable to regulate this absorption through the skeleton. Only a mechanism of this order could explain the symptoms of tetany if the patient had normal parathyroids on the right side" (4).

The assumption that three *normal* parathyroid glands are inadequate for the prevention of hypocalcemia is not the only alternative to an untenable assumption of their absence. It is conceivable that these glands are functionally inadequate because of involution and atrophy. Some indications of involution of the parathyroids in experimental hyperparathyroidism were obtained by us in dogs (7). Involution, atrophy, and hypofunction of the other parathyroid glands may be conceived of as an effect of the hyperfunction of the adenomatous gland. After termination of a condition of hyperparathyroidism by the removal of the parathyroid adenoma, there will therefore be a lack of active parathyroid secretion, and hypocalcemia will follow. In some of these cases the parathyroid glands may have been potentially active, the glands gradually recovering their activity after the removal of the adenoma. This is indicated by their eventual recovery with a return of serum calcium to normal (4-6).

It seemed desirable to establish the regularity of the development of hypocalcemia under varying methods of prolonged administration of parathormone, preliminary to a more detailed study of the mechanism involved.

Daily Treatment with Moderate Parathormone Doses—Three guinea pigs, 2 to 7 days old (weighing 80 to 130 gm.), were injected with daily parathormone doses increasing gradually from 1 to 20 units. The latter dose was administered for the last 70 to 90 days of the experiment. Serum calcium and phosphorus were determined first after 9 to 12 weeks on 20 units daily, and 24 hours after the last injection. Injections were continued for 2 days, and 5 days later the experiments were terminated and serum calcium and phosphorus again determined. In one animal treated in a similar fashion the serum calcium and phosphorus were determined 2 days after the last injection. Table II shows the results obtained.

The values obtained after these long periods of parathormone treatment, even at the 24 hour interval after the last injection, seemed to show a tendency to hypocalcemia; 2 and 5 days after the last injection hypocalcemia was definite in three of the four animals. The phosphorus values were higher after the longer intervals than after the shorter.

TABLE II
Effect of Daily Treatment with Small Doses of Parathormone

Guinea pig No.	Days after last injection	Serum Ca	Serum P
		<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
947	2	8.8	
950	1	9.1	3.4
	5	7.9	5.9
954	1	9.9	5.2
	5	10.0	6.5
956	1	9.8	4.8
	5	8.6	7.2

The shorter intervals preceded the longer. The figures in bold-faced type represent the values obtained after the longer intervals.

TABLE III
Effect of Large Intermittent Doses of Parathormone

	Days after injection	Serum P	Serum Ca
		<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
Young guinea pig	13	8.6	6.2
	4	11.0	8.0
" " "	13	10.0	7.1
	4	10.0	6.6
Adult " "	13	7.6	4.6
	4	10.1	5.9

The longer intervals preceded the shorter. The figures set in bold-faced type represent the values obtained after the longer intervals.

Large Intermittent Doses—In another group large intermittent doses were employed. Into two young guinea pigs were injected doses gradually increasing from 60 to 140 units at intervals of about 9 days. The duration of the experiment was 115 days. Blood was taken near the termination of the experiment, 13 days after an injection of 140 units, and again 10 days later (and 4 days

after the last injection). An adult female was given eight injections of 200 units each at intervals of 10 days. Blood was drawn for analysis 13 days after the last injection. Two more injections were administered and the experiment was terminated, serum calcium and phosphorus being determined 4 days after the last injections. The results are shown in Table III.

In these animals, with the larger doses employed, the effects of parathormone, including hypercalcemia and hyperphosphatemia, were assumed on the basis of our previous experience to persist longer; the 4 day interval would witness the beginning of recovery from those effects. (One animal included in this series, but not reported above, had died of acute hyperparathyroidism 3 days after the last injection of 140 units on the 90th day of the experiment.) At the 13th day the immediate after effects of the dose would have passed, and the recovery would have progressed, with considerable repair of the bone lesions. These assumptions have been confirmed by histological study (2).

Individual differences may be observed as to the time at which parathormone effects and recovery therefrom begin and end. In one guinea pig recovery may have proceeded faster than in the other two. This may explain the absence of hyperphosphatemia on the 4th day and of hypocalcemia on the 13th day.

SUMMARY AND DISCUSSION

The above data showed fairly consistent development of hypocalcemia in guinea pigs after a long course of treatment with parathormone, with some variability in the degree of hypocalcemia and the time of its incidence. The hypocalcemia was observed as early as 2 days after a moderate dose and as late as 13 days after a large dose; that is, at a time when the immediate effect of the last dose had passed. Accompanying the hypocalcemia was a rise of the serum phosphorus after the discontinuance of the daily administration of parathormone, and a relative fall after the intermittent administration. The guinea pigs received a normal diet, adequate as to calcium content, and therefore another explanation must be sought than the one suggested for the hypocalcemia in a dog on a low calcium diet, suffering from experimental hyperparathyroidism (8).

The most plausible explanation is as follows: During the administration of parathormone the calcium is mobilized and excreted; after the discontinuance of parathormone, the process is reversed; there is an increased tendency to redeposit calcium in the bones, and this tendency may be so great as to cause hypocalcemia. There was indeed histological evidence of bone resorption and repair after the discontinuance of parathormone treatment (2, 9). However, other factors in the development of hypocalcemia after hyperparathyroidism are conceivable.

Changes in Phosphorus Balance—As is well known, administration of phosphates causes hypocalcemia. Changes in the phosphorus balance having a similar effect may well have been the first produced after the withdrawal of parathormone. Phosphorus retention and hyperphosphatemia (with a positive calcium balance and hypocalcemia) were found after complete parathyroidectomy in dogs (10). Phosphorus retention was also observed after discontinuance of parathormone in essentially normal human cases (11).

Functional Changes in Parathyroid Glands.—It is also possible that during the prolonged administration of parathormone the parathyroid glands suffer involution and atrophy with resulting hypofunction. The experimental findings are consistent with this assumption. However, definite proof of hypofunction of the parathyroid glands during and after experimental hyperparathyroidism is still to be produced.

CONCLUSIONS

Hypocalcemia observed several days after the discontinuance of prolonged parathormone treatment is probably due to the rapid redeposition of calcium in the previously depleted bones, associated with changes in the phosphorus balance. It is also suggested that a temporary hypofunction of the parathyroid glands may have been caused by the prolonged parathormone treatment; immediately after the termination of experimental hyperparathyroidism, the hypofunction of the parathyroid glands contributes to the production of the complex which is characteristic of the period of recovery—hypocalcemia, hyperphosphatemia, and a positive calcium and phosphorus balance. The same explanations are suggested for the analogous developments following the removal of

parathyroid adenomas in osteitis fibrosa cystica (Recklinghausen's disease).

The generous cooperation of Eli Lilly and Company, who supplied most of the parathormone used in these experiments, is acknowledged.

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THE RELATION OF THE CONCENTRATION OF CALCIUM TO THAT OF PROTEIN AND INORGANIC PHOSPHATE IN THE SERUM

By ISIDOR GREENWALD

*(From the Department of Chemistry, New York University and Bellevue
Hospital Medical College, New York)*

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That some of the calcium in plasma or serum is bound to protein was suggested 60 years ago (1), but the first experimental evidence in favor of such a view seems to have been furnished by Rona and Takahashi (2). Additional work, reviewed by Peters and Eiserson (3) and by Loeb (4) has made this quite certain, and the more recent work has been directed to the determination of the amount of calcium combined with protein. With that, we are not at present concerned. We wish merely to consider the numerical relations between the amounts of calcium, inorganic phosphate, and protein in the serum.

From some data of their own and from some of those of Salvesen and Linder (5), Hastings, Murray, and Sendroy (6) derived a relation between calcium and protein which, expressed in the terms to be used in this paper, is

$$\text{Ca} = 5.6 + 0.56 \text{ protein}$$

and in which Ca is expressed in mg. and protein in gm. per 100 cc.

Using group averages of their own data and those of Salvesen and Linder, Peters and Eiserson (3) found that the equation

$$\text{Ca} = 6.0 + 0.56 \text{ protein}$$

held if the average inorganic phosphate content was less than 5 mg. of phosphorus per 100 cc. of serum. Including averages of the results of analyses of sera in which the amount of inorganic phosphate was greater, they derived the equation

$$\text{Ca} = 7 + 0.566 \text{ protein} - 0.255 \text{ P}$$

in which calcium and phosphorus are expressed as mg. per 100 cc. and protein as gm. per 100 cc.

Peters and Eiserson state that this formula will yield a value for calcium that agrees with the observed value, with an average error of ± 0.7 mg. per 100 cc. However, in eight instances, the discrepancy was more than 2 mg. per 100 cc. The greatest differences were observed in material from patients with uremia. In view of the changes in the concentration of other electrolytes and of pH in uremic sera, these discrepancies are, as Peters and Eiserson state, not surprising. Nevertheless, one may seriously question the usefulness of a formula which yields calculated values having an average error of ± 0.7 mg. per 100 cc. After all, the difference between a normal concentration of calcium and that at which tetany occurs is only 3 mg. per 100 cc., and the difference between the normal concentration and that at which definite symptoms referable to the hypercalcemia occur is scarcely greater. If the *average* error was ± 0.7 mg., the error must frequently have been much greater, say half the distance between normal conditions and tetany.

In a recent series of papers, Turner (7) has presented the results of detailed analyses of sera obtained from patients with pellagra and from a smaller number of control cases. Turner compared the observed calcium concentrations with those calculated by the formula of Peters and Eiserson and concluded that, "As a group, the pellagrins showed a tendency for observed values to be higher than the calculated figures, whereas the patients without pellagra showed very little difference between calculated and determined values" ((7) p. 97).

However, examination of the figures for the twenty-nine specimens obtained from twenty-four non-pellagrous cases showed a variation of the observed from the calculated values of from -29 to $+22$ per cent, and of the twenty-nine analyses reported, only ten agreed with the calculated value to within 5 per cent and only eighteen to within 10 per cent.¹

¹ The data have been expressed in terms of per cent deviation from the calculated because this was the method employed by Turner. It would in our opinion, be preferable to give the deviation of the *calculated* from the *observed* in terms of mg. This has been done in the consideration of all the other data.

It seemed that some other formula might be more satisfactory. That of Hastings, Murray, and Sendroy and its modification by Peters and Eiserson for sera containing less than 5 mg. of inorganic phosphorus per 100 cc. were tried and found to be no better than the one containing the factor for phosphorus. When, however, the observed values for calcium and protein were plotted, it was found that almost all of them fell quite close to the line

$$\text{Ca} = 5 + 0.875 \text{ protein}$$

The distribution of the variations of the observed values from those calculated by the four different formulæ is shown in Table I.

TABLE I
Distributions of Variations of Observed Values for Calcium from Those Calculated

Data of Turner (see foot-note 1); 29 specimens.

Agreement	No. of cases			
	Equation 1	Equation 2	Equation 3	Equation 4
Within 2 per cent	6	6	2	15
“ 5 “ “	10	9	8	22
“ 8 “ “	16	11	16	26*
Average deviation, mg	±0.73	±0.92	±0.76	±0.35

Equation 1, $\text{Ca} = 7 + 0.566 \text{ protein} - 0.255 \text{ P}$

“ 2, “ = $5.6 + 0.56$ “

“ 3, “ = $6.0 + 0.56$ “

“ 4, “ = $5 + 0.875$ “

* The remaining three were -26, +16, and -18 per cent.

It is perfectly obvious that the formula $\text{Ca} = 5 + 0.875 \text{ protein}$ gives values that are much more closely in agreement with those observed than do the others.

Applying the new equation to the 137 specimens from the forty-eight cases of pellagra, we find that the deviation of the observed values from those calculated is

Less than 1 per cent in 11 samples
“ “ 5 “ “ “ 57 “
“ “ 10 “ “ “ 96 “
“ “ 20 “ “ “ 130 “

No attempt has been made by the present writer to calculate the differences between the observed values for calcium in these specimens and those calculated by any other formula, but reference to Fig. 1 of Turner's paper ((7) p. 92) shows that the observed values varied rarely less than 10 per cent, generally more than 20 per cent, and frequently more than 30 per cent from those calculated by the formula of Peters and Eiserson.

The seven specimens in which values differed more than 20 per cent from those calculated from the equation $Ca = 5 + 0.875$ protein were obtained from

Case No	per cent
8	+24 (only specimen)
9	+21 (but only -8, -3, -8, -0.2 per cent in other specimens)
23	+29 (" " -10 per cent in another specimen)
32	+21 (" " +13, +10, +12, +4 per cent in other specimens)
33	-49 (" " +9, -8, +1 per cent in other specimens)
34	+48 (" " +2, +2, +11, +15 per cent in other specimens)
35	+20 (" " -0.4 per cent in another specimen)

The two greatest deviations are probably due to experimental error, for they are due to observed values of 6.0 and 14.3 mg. per 100 cc., whereas other determinations made 2 weeks earlier and later on the same individuals gave 10.8 and 11.1 mg. for the former and 11.0 and 11.1 mg. for the latter.

In consideration of the fact that the formula $Ca = 5 + 0.875$ protein was derived from twenty-nine analyses and was applied to 137, the agreement would appear to be quite satisfactory, indicating that there is probably no particular disturbance of calcium concentration in the serum in pellagra. However, we are not now primarily concerned with this question but the more general subject of the relation between calcium, phosphate, and protein concentrations in serum.

Merritt and Bauer (8) have published a large number of values for calcium and protein and a smaller number for inorganic phosphate. When the values for calcium were plotted against those for protein, it was found that the slope of the locus for the values of calcium was about 0.875 protein, but that it cut the ordinate, protein = 0, at about 3.7 and not at 5, as in Turner's series.

Before proceeding to a consideration of the comparisons between

the observed and calculated values, it may be in order to discuss the nature of the classification adopted. It will be generally recognized that 0.2 mg. represents the limit of error of calcium determinations in serum, even with careful technique. Moreover, considering the nature of the determinations involved in the calculations, it is believed that differences of 0.5 mg. between the calculated values and those observed may be expected, no matter

TABLE II

Differences between Observed and Calculated Values for Calcium
Data of Merritt and Bauer.

Difference	No. of cases in which inorganic phosphate						
	Was determined Equation No.				Was not determined Equation No		
	1	2	3	4	2	3	4
<i>mg.</i>							
0.2 or less	12	11	10	14	7	10	11
0.21-0.50	15	19	7	19	22	10	13
0.51-1.0	11	10	18	7	9	22	15
1.01-2.0	5	3	8	3	8	4	7
>2.0	0	0	0	0	1	1	1
Maximum deviation, <i>mg.</i>	1 67	1 29	1.69	1.53	2.05*	2.45†	2.16*
Average deviation, <i>mg.</i>	±0.48	±0.50	±0.63	±0.40	±0.53	±0.49	±0.54

Equation 1, Ca = 7 + 0.566 protein - 0.255 P

" 2, " = 5.6 + 0.56 "

" 3, " = 6.0 + 0.56 "

" 4, " = 3.7 + 0.875 "

* Ca = 6.75, protein = 5.72 (tuberculosis).

† Ca = 9.50, protein = 9.10 (tuberculosis).

what formula may be chosen. A variation of 1 mg. may be regarded as the limit of the physiological and, probably, sufficient to indicate a disturbance in calcium metabolism. Accordingly, the deviations of the calculated values from the observed have been classified as (1) 0.2 mg. or less, (2) 0.21 to 0.50 mg., (3) 0.51 to 1.0 mg., (4) more than 1.0 mg.

In Table II there is presented a summary of the deviations of

the calculated values from the observed, expressed in terms of mg. of calcium per 100 cc. of serum and also giving the average deviation. The figures show very clearly that the values calculated from the equation $\text{Ca} = 3.7 + 0.875 \text{ protein}$ correspond more closely to the observed values than do those calculated by any of the other equations.

There have recently appeared the results of ten analyses by Greene and his associates (9). These give the results summarized in Table III. The agreement with the equation $\text{Ca} = 3.7 + 0.875 \text{ protein}$ is about as good as that with the formula of Peters and

TABLE III
Differences between Observed and Calculated Values for Calcium
Data of Greene, Bollman, Keith, and Wakefield.

Difference	No. of cases			
	Equation 1	Equation 2	Equation 3	Equation 4
<i>mg.</i>				
0.2 or less	2	0	4	1
0.21-0.50	2	4	4	1
0.51-1.0	3	4	1	5
>1.0	3	2	1	3
Maximum deviation, <i>mg.</i>	2.09	1.49	1.09	2.30
Average " "	± 0.75	± 0.70	± 0.25	± 0.91

Equation 1, $\text{Ca} = 7 + 0.566 \text{ protein} - 0.255 \text{ P}$

" 2, " = $5.6 + 0.56$ "

" 3, " = $6.0 + 0.56$ "

" 4, " = $3.7 + 0.875$ "

Eiserson, though not as close as with the equation $\text{Ca} = 0.56 \text{ protein} + 6.0$.

In Table IV we present a summary of a comparison of the observed values for calcium in serum reported by Salvesen and Linder with those calculated by the four different equations that have been proposed. It will be seen that the agreement is rather better in the case of the equation $\text{Ca} = 3.7 + 0.875 \text{ protein}$ than in any of the others.

We have excluded from consideration in Table IV those instances in which the plasma proteins were not determined on the same

day as the serum calcium and phosphate, as well as the four samples which showed very large amounts of inorganic phosphate. In Table V there have been collected the figures for the phosphate, protein, and calcium content of these four samples and of all others with an inorganic phosphate content of 6.0 mg. or more per 100 cc. that are to be found in any of the reports considered in this paper. There have also been added the figures obtained by Blackfan and Hamilton (10) and by Boyd, Courtney, and MacLachlan (11) in the analyses of nephritic sera. Table V also presents the

TABLE IV

Differences between Observed and Calculated Values for Calcium

Data of Salvesen and Linder. Inorganic phosphate less than 10 mg. per 100 cc.

Difference	No. of cases			
	Equation 1	Equation 2	Equation 3	Equation 4
<i>mg.</i>				
0.2 or less	12	10	9	12
0.21-0.50	10	14	6	12
0.5-1.0	7	7	16	7
>1.0	3	1	1	1
Maximum deviation, <i>mg.</i>	1.2	2.0*	2.4*	1.7*
Average " "	±0.42	±0.41	±0.57	±0.35

Equation 1, $\text{Ca} = 7 + 0.566 \text{ protein} - 0.255 \text{ P}$

" 2, " = $5.6 + 0.56$ "

" 3, " = $6.0 + 0.56$ "

" 4, " = $3.7 + 0.875$ "

* Inorganic phosphate = 9 mg. per 100 cc.

differences between the figures calculated by the four equations discussed in this paper and the observed values for the calcium content. It is obvious that those calculated from the equation $\text{Ca} = x + 0.875 \text{ protein}$, in which $x = 5$ for Turner's series and 3.7 for all the others considered, are in as close agreement with the observed values as are those calculated by the equation of Peters and Eiserson.

It may be objected that this good agreement between the observed values and those calculated by the use of the equation Ca

TABLE V

Comparison of Observed and Calculated Values of Calcium in Sera Containing 6.0 Mg. or More of Inorganic Phosphorus per 100 Cc. and in a Few Other Specimens Not Included in Tables I to IV

Observer	Specimen No.	P	Protein	Ca	Difference between observed values and those calculated by			
					Equation 1	Equation 2	Equation 3	Equation 4
		mg. per cent	per cent	mg. per cent	mg.	mg.	mg.	mg.
Turner	17 C	6.3	6.75	9.5	-0.3	-0.1	+0.3	+1.4
	17 D	12.0	5.85	8.5	-1.2	+0.4	+0.8	+1.6
	23 B	6.7	4.87	11.9	-3.8	+1.8	+2.2	-2.6
	25 B	6.2	6.17	10.76	-1.85	+0.91	+1.31	-0.36
	27 C	6.3	6.14	10.6	-1.7	+0.8	+1.2	-0.2
	46 A	13.3	7.07	10.7	-3.1	+0.3	+0.7	+0.5
	46 B	11.6	6.38	10.1	-2.4	+0.1	+0.5	+0.5
Salvesen and Linder	9 B	8.0	5.87	8.8	-0.6	+0.1	+0.5	0.0
	12 C	6.3	4.59	9.2	-0.2	+1.0	+1.4	-0.5
	12 D	6.7	4.37	7.8	-0.1	+0.3	+0.7	-0.3
	13 A	8.0	4.52	7.7	-0.2	+0.4	+0.8	0.0
	13 D	7.3	5.15	8.1	-0.1	+0.4	+0.8	+0.2
	13 E	9.0	4.87	6.3	+1.2	+1.0	+1.4	+1.7
	14 B	14.1	6.15	8.0	-1.1	+1.0	+1.4	+1.1
	14 C	18.8	6.87	7.3	-1.2	+2.1	+2.5	+2.4
	14 D	20.5	6.29	6.8	-1.5	+2.3	+2.7	+2.4
	15	24.0	5.95	7.2	-3.0	+1.7	+2.1	+1.7
Blackfan and Hamilton	C	4.1	4.7	8.2	+0.4	0.0	+0.4	-0.4
	P	6.55	3.8	7.6	-0.1	+0.1	+0.5	-0.6
	N	6.2	4.3	8.0	-0.1	0.0	+0.4	-0.5
	P	4.5	2.8	6.6	+0.8	+0.6	+1.0	-0.4
	P	5.2	2.6	6.8	+0.3	+0.3	+0.7	-0.8
	G	3.8	7.8	9.0	+1.4	+1.0	+1.4	+1.5
Boyd, Courtney, MacLachlan	A. J.	5.5	6.0	9.2	-0.2	-0.2	+0.2	-0.2
	A. C.	3.8	6.1	9.4	+0.1	-0.4	0.0	-0.4
	A. C.	3.0	6.5	9.8	+0.1	-0.6	-0.2	-0.8
	E. D.	6.3	5.0	7.2	+1.0	+1.2	+1.6	+0.9
	E. D.	6.0	5.0	8.8	-0.5	-0.4	0.0	-0.7
	I. T.	6.5	6.55	9.0	0.0	+0.3	+0.7	+0.4
Average deviation					±1.0	±0.69	+0.99	±0.87

Equation 1, $\text{Ca} = 7 + 0.566 \text{ protein} - 0.255 \text{ P}$

" 2, " = $5.6 + 0.56$ "

" 3, " = $6.0 + 0.56$ "

" 4, " = $x + 0.875$ " $x = 5$ in Turner's series and

3.7 in all the others

= $x + 0.875$ protein can be obtained only by the use of varying values of x . This is not surprising. The values for the composition of normal serum that are obtained in different laboratories vary considerably. Thus, those reported for calcium from Turner's laboratory are quite definitely higher than those reported by most others. This may be due to differences in technique or, in this particular instance, to the influence of climate. Whatever the cause for such variations may be, it would seem that the equation $\text{Ca} = x + 0.875$ protein is of quite general validity, provided that sufficient control estimations are made to establish the value of x .

This is not meant to imply that it is the most accurate formula that can be derived or that no other relation between protein and calcium can be established. In fact, it is quite obvious from the results presented in Tables I to V, or from any attempt to plot the values for calcium and phosphorus found in the literature, that almost any number of equations can be derived, all of which will yield fairly good agreement with the observed values.

No equation derived from analyses of sera of normal solute and hydrogen ion concentration can be expected to be closely applicable to uremic conditions, but the failure of the usual correlation between calcium and protein concentrations is, apparently, not due merely to the accumulation of phosphate.

Since practically all of the calcium in the body is present as calcium phosphate, it is obvious that any disturbance in the metabolism of calcium must affect that of phosphate, but there may be changes in the latter that have no direct relation to the former. Moreover, metabolism may be disturbed without perceptible changes in the concentration in the blood. As a rule, plasma calcium concentration and calcium metabolism, phosphate concentration and phosphate metabolism are interrelated but the concentration of either calcium or phosphate may be affected without, necessarily, involving the other.

The idea that there is a relation between calcium and phosphate concentrations seems to derive its origin from the experiments of Binger (12), who found that the calcium content of the serum of dogs was diminished after the injection of large volumes of sodium phosphate solution. In the absence of all consideration of the effect of the large volumes introduced in diluting the body fluids or of any control experiments with solutions of other sodium salts, it is

difficult to understand why Binger's experiments should have been considered as evidence that the phosphate ion had any specific action in lowering the calcium concentration. That this is not the case was indicated by the author's analysis of Binger's results as well as by his own experiments (13), in which the injection of sodium sulfate solutions produced quite as marked decreases in the concentration of calcium in the serum as did injections of sodium phosphate.

In this connection, the experiments of Salvesen, Hastings, and McIntosh (14) are of great interest. They administered sodium phosphate to dogs, by mouth, and found that when they succeeded in lowering the concentration of calcium in the serum, there was a coincident increase in the phosphate concentration. Nevertheless, as they expressly point out, similar and even greater increases in phosphate concentration were observed in some experiments in which there was no, or but little, decrease in the concentration of calcium.

In conclusion, it may be observed that attempts to derive an equation for the relation between the concentrations of calcium, phosphate, and protein in the sera of dogs, based upon analyses in the author's laboratory or found in the literature, have been unsuccessful. It may be that the greater heterogeneity of the material is responsible for this failure.

Addendum—Since the above was written, two additional papers containing significant material have appeared. One of these (15) deals with the changes in the calcium, phosphorus, and protein content of the sera of normal individuals under a variety of conditions. The authors conclude, "In such temporary fluctuations the serum calcium does not always vary inversely with the serum inorganic phosphorus, although there is some tendency for it to do so. Temporary changes in serum protein are not always associated with corresponding variations in the serum calcium." The authors of the other paper (16) who used sera obtained from children state that, "No constant relationship was observed between the level of serum calcium and serum protein or serum inorganic phosphorus."

If the data for calcium and protein content reported in these papers are plotted on coordinate paper, it is found, as has already been mentioned on p. 559, that a very great number of straight lines, having very different slopes, may be drawn through the points. If the equation $\text{Ca} = 0.875 \text{ protein} + x$ is believed to be correct, x = about 6.4 in the case of newly born infants, 6.2 in those from 1 to 10 months old, and 5 in children from 1 to 16 years old. The sera of the adults of the other series yield a value of about

3.9. If, however, the equation $\text{Ca} = 0.56 \text{ protein} + x$, derived by Hastings, Murray, and Sendroy, is employed, $x = 8.3$ for newly born infants, about 8 for those from 1 to 10 months old, about 7 for children from 1 to 16 years old, and about 5.8 for adults, thus agreeing with the values found by Hastings, Murray, and Sendroy and by Peters and Eiserson. Whether or not these differences in the value of x are related to differences in the character of the plasma proteins or to changes in parathyroid activity or to intake of vitamin D remains to be determined.

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THE COMPARATIVE RATES OF HYDROLYSIS OF ADENYLIC, GUANYLIC, AND XANTHYLIC ACIDS

BY P. A. LEVENE AND A. DMOCHOWSKI*

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York)

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The ultimate object of the present investigation was the study of the structure of the phosphoric ester of *d*-ribose entering into the structure of ribonucleotides derived from ribonucleic acids. It has been assumed that the ribophosphoric acid derived from inosinic acid differs from that derived from adenylic acid which in turn had been derived from ribonucleic acid. The conclusions to this effect suggested first by Levene and Yamagawa¹ were later corroborated by Embden and Schmidt.² They were based on the difference in the rates of hydrolysis of the phosphoric acid residues from the two nucleotides. In order to reach a conclusion as to the details of the differences in the structure of the ribophosphoric acids of the two types it is necessary to be in possession of the free ribophosphoric acid derived from ribonucleic acid.

In a preliminary note recently published by Schmidt³ the announcement was made that it was possible to prepare the desired ribophosphoric acid by means of a specific enzyme. Our own efforts to prepare ribophosphoric acid from guanylic acid through the action of nucleosidase thus far have been unsuccessful, inasmuch as the enzyme potent for nucleosides is incapable of separating the purine base from nucleotides. In an earlier paper Levene and Weber⁴ recorded the fact that their best preparation of nucleosidase was not capable of separating the purine or the pyrimidine

* Fellow of The Polish Institute for Cultural Relations, Warsaw, Poland.

¹ Levene, P. A., and Yamagawa, M., *J. Biol. Chem.*, **43**, 323 (1920).

² Embden, G., and Schmidt, G., *Z. physiol. Chem.*, **181**, 130 (1929).

³ Schmidt, G., *Klin. Woch.*, **10**, 165 (1931).

⁴ Levene, P. A., and Weber, I., *J. Biol. Chem.*, **60**, 707 (1924).

bases from the molecule of nucleic acids. It has now been found that the nucleosidases also lack the power to separate the bases from guanylic and adenylic acids.

However, Levene and Jorpes⁵ had observed that in nucleotides the rate of acid hydrolysis of both the base and the phosphoric acid residue is modified by small changes in the structure of the base. Thus, in the case of dihydrouridinephosphoric acid, the rate of hydrolysis is of the same order of magnitude as in that of purine nucleotides, whereas the rate of removal of the phosphoric acid residue from uridinephosphoric acid is very low. By analogy, it seemed possible that the rates of hydrolysis of guanylic and xanthylic acids might be different. Indeed, it was found that in a low hydrogen ion concentration, such as exists in a 1 per cent solution of guanylic or of xanthylic acid in water at 50°, the former remains practically unaltered, whereas the latter loses the base to the extent of 45 per cent, leaving the phosphoric acid residue almost intact. Thus a way has been found for the preparation, by chemical means, of *d*-ribosephosphoric acid from guanylic acid.⁶

In the present communication there are discussed three distinct phases of the problem: (1) the preparation of guanylic and xanthylic acids; (2) the effect of nucleosidase on nucleosides and on purine nucleotides; (3) the hydrolysis of guanylic and of xanthylic acids.

EXPERIMENTAL

Preparation of Sodium Guanylate

The amorphous sodium salt suffices for the present purpose. It is prepared by a modification of the method of Steudel and Peiser.⁷

1000 cc. of 5 per cent solution of sodium hydroxide are brought to the boil and in the hot solution 250 gm. of nucleic acid (Boehringer und Sohn) are dissolved. The solution is allowed to stand on a boiling water bath for 8 minutes after which it is freed from a

⁵ Levene, P. A., and Jorpes, E., *J. Biol. Chem.*, **81**, 575 (1929).

⁶ This work is published before the *d*-ribosephosphoric acid has been isolated owing to the termination of the visit of Dr. Dmochowski to The Rockefeller Institute.

⁷ Steudel, H., and Peiser, E., *Z. physiol. Chem.*, **114**, 201 (1921); **120**, 292 (1922).

sediment by centrifugalization. The clear solution is neutralized with acetic acid and allowed to stand in the cold overnight. The precipitate thus formed is centrifugalized, washed with a cold 20 per cent solution of sodium acetate, and redissolved by boiling with 500 cc. of a 20 per cent solution of sodium acetate. On cooling, the sodium salt of guanylic acid forms a semigelatinous precipitate. The operation is repeated several times.

The substance prepared in this manner had the following composition.

5.020 mg. substance	5.321 mg. CO ₂ and 2.080 mg. H ₂ O.
5.240 " "	0.757 cc. N ₂ (27.5°, 755 mm.).
5.430 " "	27.345 mg. P molybdate.
C ₁₀ H ₁₂ N ₅ PO ₃ Na ₂ .	Calculated. C 29.48, H 2.95, N 17.19, P 7.61
	Found. " 28.90, " 3.55, " 16.31, " 7.31

In a 5 per cent solution of sodium hydroxide it has the following rotation.

$$[\alpha]_D^{20} = \frac{-1.72^\circ \times 100}{1 \times 3} = -57.3^\circ$$

On hydrolysis of 2 gm. of the substance with hydrogen chloride in methyl alcohol the yield of guanine chloride was 1.85 gm.

Preparation of Xanthylic Acid

Free xanthylic acid has not hitherto been described although Knopf^a described its brucine salt. The procedure for its preparation is practically the one which has been employed by Levene and Jacobs for conversion of guanosine into xanthosine and which Knopf used for the preparation of xanthylic acid. 20 gm. of the sodium salt of guanylic acid are dissolved in 20 volumes of water and to the solution 120 cc. of glacial acetic acid are added. Into the solution 300 cc. of a 30 per cent potassium nitrite solution are allowed to flow in a very slow stream and the mixture is stirred mechanically for about 6 hours, at the end of which time the reaction product has lost entirely its sticky viscous character. The reaction product is freed from the remaining traces of guanylic acid by filtration. The xanthylic acid is then precipitated by a 25 per cent solution of neutral lead acetate. The salt is suspended

^a Knopf, M., *Z. physiol. Chem.*, **92**, 159 (1914).

in water and freed from lead by means of hydrogen sulfide. The acid is reconverted into the lead salt. The operation is repeated several times after which the solution of free acid is concentrated under diminished pressure to a smaller volume in a water bath at 35°.

The acid then settles out in the form of a white powder consisting of microscopic striated balls. By repeating the operation further it will doubtless be possible to obtain the substance in perfectly crystalline form.

The substance had the following composition.

4.810 mg. substance · 5.807 mg. CO₂ and 1.53 mg. H₂O.

0.0896 gm. required 9.93 cc. 0.1 N HCl.

4.495 mg. substance: 26.615 mg. Mg₂P₂O₇.

C₁₀H₁₃N₄PO₉. Calculated. C 32.95, H 4.12, N 15.38, P 8.51

Found. " 32.92, " 3.55, " 15.62, " 8.60

The rotation of the substance in 5 per cent sodium hydroxide solution was the following.

$$[\alpha]_D^{20} = \frac{-1.25^\circ \times 100}{1 \times 3} = -41.66^\circ$$

Action of Enzymes

The crude enzyme was prepared from pig intestines according to the directions of Levene and Weber.⁴ This crude material contained both enzymes, the nucleophosphatase and nucleosidase.

The nucleosidase activity can be seen from Table I.

In Table II is shown the action of the same enzyme on the sodium salt of guanylic acid. It can be seen from this table that when the enzyme is used in a moderate quantity the phosphatase action proceeds at a higher rate than the cleavage of the base. When a large excess of the enzyme is used the two reactions proceed at approximately equal rates.

Efforts were then made either to separate the two enzymes or to find conditions under which the reaction of the phosphatase would be retarded. All possible conditions were not exhausted but thus far whenever the nucleophosphatase action was inhibited, or whenever the nucleophosphatase was removed, the enzyme material failed to remove the base from the nucleotides even when it was active on nucleosides.

In Table III is given the result of the action of an enzyme which

TABLE I
Adenosine

Time	$\frac{1}{10}$ N thiosulfate used	Difference	Reducing ribose	
200 mg. adenosine in 30 cc. H ₂ O + 20 cc. veronal buffer (pH, 7.0) + 250 mg. enzyme (pig)				
hrs.	cc.	cc.	mg.	per cent
0	0.36		0	
4	1.20	0.84	28.0	26.2
22	1.90	1.54	51.2	50.3
50 mg. adenosine in 10 cc. H ₂ O + 15 cc. phosphate buffer (pH, 7.0) + 150 mg. enzyme (pig)				
0	0.50		0	
2	0.84	0.34	5.7	23.0
22	2.0	1.50	25.0	99.0

TABLE II
Guanylic Acid (Sodium Salt)
Crude Enzyme

Time	$\frac{1}{10}$ N thiosulfate		Reducing ribose		Phosphorus (as PO ₄ ")		
	Found	Increase			Found	Increase	
250 mg. (Na ₂ -guanylate) + 30 cc. H ₂ O + 20 cc. veronal buffer (pH, 7.0) + 250 mg. enzyme							
hrs.	cc.	cc.	mg.	per cent	mg.	mg.	per cent
0	0.1	0	0		14.44	0	0
4	0.31	0.21	7.0	7.8	15.75	1.3	7.2
22	0.46	0.36	12.0	13.3	22.33	7.9	43.3
250 mg. (Na ₂ -guanylate) + 30 cc. H ₂ O + 20 cc. veronal buffer (pH, 7.0) + 1000 mg. enzyme							
0	0.28	0	0		51.6	0	0
4	0.78	0.5	16.7	17.5			
22	2.43	2.15	71.7	78.0	65.0	13.4	73.6

was prepared from the crude enzyme by purifying according to the directions of von Euler and Brunius.⁹ This enzyme had a higher nucleophosphatase than nucleosidase potency.

⁹ von Euler, H., and Brunius, E., *Ber. chem. Ges.*, **60**, 1584 (1927).

After dialysis the same enzyme solution used in a large excess apparently removed a larger proportion of base than of phosphoric acid (Table IV). Whether the result was due to the dialysis or to the differences in the proportion of enzyme to substrate cannot as yet be stated. At all events the differences in the rates of action of the two enzymes were not significant.

TABLE III
Adenylic Acid

Enzyme from Pig Kidney Precipitated with Acetone

250 mg. adenylic acid + 30 cc. H₂O + 20 cc. veronal buffer (pH, 7.0) + 250 mg. enzyme.

Time	α_0 N thiosulfate		Reducing ribose		Phosphorus (as PO ₄ ")		
	Found	Increase			Found	Increase	
<i>hrs.</i>	<i>cc.</i>	<i>cc.</i>	<i>mg.</i>	<i>per cent</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
0	0.1	0	0	0	13.3	0	
4	0.29	0.19	6.3	6.2	14.75	1.45	6.8
22	0.38	0.28	9.3	9.1	23.62	10.3	48.1

TABLE IV

Guanylic Acid (Sodium Salt)

Enzyme from Pig Kidney Precipitated with Acetone and Dialyzed for 24 Hours

250 mg. (Na₂-guanylate) in 30 cc. H₂O + 20 cc. veronal buffer (pH, 6.0) + 1600 mg. enzyme.

Time	α_0 N thiosulfate		Reducing ribose		Phosphorus (as PO ₄ ")	
	Found	Increase			Found	Increase
<i>hrs.</i>	<i>cc.</i>	<i>cc.</i>	<i>mg</i>	<i>per cent</i>	<i>mg.</i>	<i>per cent</i>
42	2.72	2.72	21.0	98.0	15.5	85.6

No reduction and no phosphorus in dialyzed enzyme.

Purification of the enzyme by aluminium hydroxide and by colloidal iron according to the procedure of Levene and Weber⁴ led to enzyme preparations that were very active when tested on nucleosides, but were totally inactive with regard to adenylic and guanylic acids.

TABLE V
Adenylic, Guanylic, and Xanthylic Acids

Time	Adenylic acid, initial pH 3.5				Guanylic acid, initial pH 2.4				Xanthylic acid, initial pH 1.9					
	25°		50°		25°		50°		25°		50°			
	PO ₄ ³⁻ per cent	Sugar per cent	PO ₄ per cent	Sugar per cent	PO ₄ ³⁻ per cent	Sugar per cent	PO ₄ per cent	Sugar per cent	α° degrees	PO ₄ per cent	Sugar per cent	α° degrees	PO ₄ per cent	Sugar per cent
hrs.														
0	None	0.0	None	0.0	None	0.0	None	4.8	None	4.8	0.9	None	0.9	0.9
23														
47														
95	None	0.2	None	1.2	None	4.1	None	4.1	None	13.1	None	16.5	5.9	47.4

with
high

Hydrolysis of Guanylic and Xanthylic Acids by Hydrogen Ions

Several experiments were made on the hydrolysis of guanylic and xanthylic acids by *N* hydrochloric acid at temperatures of 50° and 75°. There was no appreciable difference in the rate of hydrolysis of the two substances. However, when aqueous solutions of guanylic (also of adenylic) and of xanthylic acids were hydrolyzed without the addition of mineral acid at 25° and at 50°, then an unmistakable difference was observed in the conduct of the individual substances. Whereas the first two showed but little change under these conditions, xanthylic acid lost 47.4 per cent of its base, without the liberation of any appreciable quantity of phosphoric acid. As yet it cannot be stated whether the differences in behavior were due to differences in the hydrion concentration of the solutions or to the differences in the stabilities of the individual nucleotides.

The results of the observations are given in Table V.

The reducing values were determined by the Hanes¹⁰ modification of the Hagedorn-Jensen method on samples taken directly from the mixtures without treatment. The results thus found were only slightly higher than those on samples which were precipitated first with mercuric acetate, then treated with H₂S, and finally aerated to remove the excess of hydrogen sulfide.

The phosphate determinations were made by precipitating the inorganic phosphate with magnesium ammonium chloride, dissolving this precipitate in dilute hydrochloric acid, and measuring the phosphate by the colorimetric method of Kuttner and Cohen.¹¹

Thus a way is given for the preparation of *D*-ribophosphoric acid entering into the structure of guanylic acid derived from ribonucleic acid.

SUMMARY

1. A method for the preparation of xanthylic acid is given.
2. The properties of free xanthylic acid are described.
3. Nucleosidase derived from pig intestinal wall is incapable of removing the base from nucleotides.
4. Xanthylic acid in aqueous solution at 50° loses in 48 hours over 40 per cent of its base without the liberation of free phosphoric acid, thus giving rise to *D*-ribophosphoric acid.

¹⁰ Hanes, C. S., *Biochem. J.*, **23**, 99 (1929). Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, **135**, 46 (1923).

¹¹ Kuttner, T., and Cohen, H. R., *J. Biol. Chem.*, **75**, 517 (1927).

CHEMISTRY OF CHROMOPROTEINS

I. ON THE CHROMOPHORIC GROUP OF THE RHODYMENIA PALMATA

By P. A. LEVENE AND A. SCHORMÜLLER*

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York)

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Considerable advances have been made in the chemistry of chromoproteins, particularly through the work of Kylin,¹ Kitasato,² and Lemberg.³ A good review of the literature can be found in the articles of the last two authors and need not be given here in great detail. In this place it suffices to mention that certain evidence was furnished in favor of the view that the prosthetic groups of the two known chromoproteins, phycoerythrin and phycocyanin, are composed of polypyrrole derivatives. The conclusion was based principally on qualitative tests. A substance isolated by Lemberg seemed to have the composition $C_{34}H_{44}O_8N_4$, which suggested a similarity to the blood and bile pigments.

The slow progress of the work on the structure of chromoproteins is due to two causes: one being the inaccessibility of the starting material, the second, the great resistance of the substance to mild treatment and its instability to drastic treatment. The present work aimed first to develop a method by which the starting material could be made more accessible and second, to isolate some characteristic components that would definitely correlate the substance to known polypyrrole derivatives.

Certain progress has been made by us in both directions. The starting material of previous workers was the crystalline protein which was subsequently digested with pepsin hydrochloric acid.

* Fellow of The Rockefeller Foundation.

¹ Kylin, H., *Z. physiol. Chem.*, **69**, 169 (1910); **76**, 369 (1912).

² Kitasato, Z., *Acta Phytochim.*, **2**, 2, 75 (1925).

³ Lemberg, R., *Ann. Chem.*, **461**, 46 (1928); **477**, 195 (1930).

The extraction of the proteins from the seaweed was never complete and was always very laborious. Yields were poor, even from the best available source, Japanese nori. From seaweed more readily accessible in America the proteins could be extracted in only infinitely small quantities. It was decided to extract the pigments after preliminary digestion of the entire seaweed inasmuch as the digestion product of the chromoproteins is soluble in amyl alcohol and in this manner can be separated from the other digestion products. The process proved quite successful, with the result that from 1 kilo of the air-dried seaweed 7 gm. of the product which Lemberg termed "phykobilin" were obtained. It was realized that this product contained the chromophoric groups of both phycoerythrin and phycocyanin. However, these two groups are structurally closely related and the phycoerythrobilin can be oxidized easily into a substance very similar to phycocyanobilin.

On purification of the phycobilin (digestion product) by neutral solvents (the details are given in the experimental part) a substance was obtained which had a composition similar to that of the substance obtained by Lemberg by more drastic treatment with hot hydrochloric acid. On treatment with methyl alcoholic hydrogen chloride our substance was esterified and a product was obtained which in its composition resembled the phycoerythrobilinic ester of Lemberg and which this author regarded as the protein-free component of the chromoprotein.

Erythrobilin ester (Lemberg)	C 61.6,	H 7.06,	N 8.85,	OCH ₃ , 6.00
" " "	"	"	"	10.40
Ester (Levene and Schormüller)	" 61.03,	" 6.66,	" 7.06,	" 10.40
	" 61.32,	" 7.14,	" 7.55	

However, our material on hydrolysis with 20 per cent hydrochloric acid for 24 hours showed an amino nitrogen content of 45 per cent of the total nitrogen and hence could not have been the chromophoric component. This fragment may be termed "chromoproteinic acid."

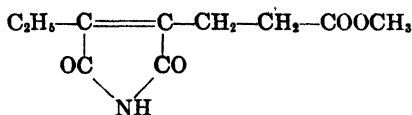
Reduction with Hydriodic Acid

On reduction with hydriodic acid a substance was obtained which had the composition $C_{17}H_{23}NO_4$. It is difficult to state

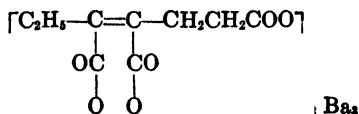
whether even this product was totally free from protein inasmuch as on hydrolysis with strong hydrochloric acid it gave a small quantity of amino nitrogen.

Oxidation with Chromic Acid

The ester on oxidation with chromic acid gave a straw-yellow liquid which, after several redistillations, had approximately the composition $C_{10}H_{13}NO_4$, thus suggesting similarity with an ester of a hematinic acid, more specifically of ethylmaleinimidopropionic methyl ester.

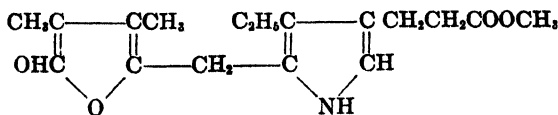


The theoretical values are C 56.87, H 6.16, N 6.6. Found, C 55.91, H 6.25, N 5.83. Inasmuch as a uniform substance could scarcely be expected, the nature of the starting material being borne in mind, the conclusion seemed warranted that the major part of the yellow liquid consisted of the above hematinic acid. This conclusion is further supported by the result of the action of barium hydroxide on this ester. As a result of this action a barium salt of a tribasic acid of the following composition was obtained.



It is significant that basic maleinimide could not be isolated from the product of oxidation.

Upon taking into consideration the latter circumstance and the analytical data on the ester that were obtained on reduction with hydriodic acid, a tentative idea can be formulated as to the composition of the ester; namely, that it is a substance containing a pyrrole and a furan ring in its structure. Thus it may be represented in the following way.



The allocations of two methyl groups and of the hydroxyl group are arbitrary. One ethyl group in place of the two methyl groups would agree equally well with the known facts.

Either of these two substances on oxidation would yield only hematinic acid and none of the basic maleinimides. The furan component on oxidation would be decomposed into volatile acids. The substance would not be readily hydrogenated, and indeed, our efforts to hydrogenate our substance were not successful.

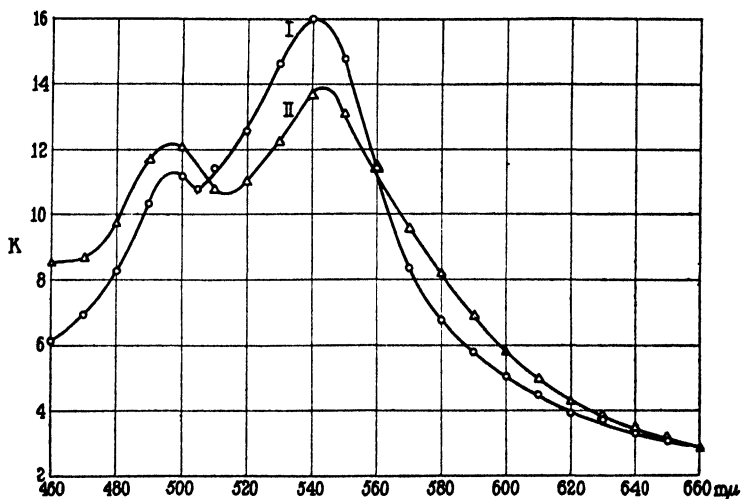


FIG. 1. Absorption curves of chromoproteinic acids.⁴ Curve I, chromoproteinic acid obtained from nori. Curve II, chromoproteinic acid obtained from *Rhodymenia palmata*. K is the extinction coefficient according to the formula $\frac{I_0}{I} = 10^{cdK}$ where c is the concentration expressed in gm. per 100 cc. and d the thickness in cm. The measurements were made with a König-Martens spectrophotometer.

We wish to emphasize again that the formula is presented only as a suggestive point of departure for further work and not as a final conclusion as to the structure of the substance.

From the curves in Fig. 1 it may be seen that the chromoproteinic acids obtained from nori and from *Rhodymenia palmata* resemble each other closely.

⁴ We wish to thank Dr. Alexandre Rothen for making the absorption curves.

EXPERIMENTAL

The dried and powdered leaves of *Rhodymenia palmata* (1 kilo) were suspended in 15 liters of water. 150 cc. of concentrated hydrochloric acid and 20 gm. of pepsin dissolved in 500 cc. of $\frac{1}{2}$ per cent hydrochloric acid were added. After standing for 6 to 8 days in the dark, the filtered aqueous solution was extracted three times with amyl alcohol in large separatory funnels. After the first extraction ammonium sulfate was added to the aqueous solution not quite to saturation and the extraction was continued. The combined amyl alcoholic extracts were concentrated under diminished pressure and decanted from the crystallized ammonium sulfate, and the solution further concentrated under reduced pressure with the addition of benzene to remove water as far as practicable.

The oily, dark violet-colored material was filtered from the ammonium sulfate which settled out, and precipitated with a large excess of ether (petroleum ether is not well adapted to this purpose).

The brownish green-colored ether solution was allowed to settle several hours and decanted from the precipitate which was collected on a large Buchner funnel. This material was washed several times with anhydrous ether, dried on the funnel, and immediately placed in a vacuum desiccator over concentrated sulfuric acid. The desiccator was kept under a continuous vacuum for 20 hours and the powdered material was dried for 24 hours in a vacuum over phosphorus pentoxide. In this manner a dark violet-colored, slightly hygroscopic powder was obtained. 1 kilo of algæ yielded about 7 gm. of coloring matter.

For analysis this product was dissolved in amyl alcohol and precipitated with anhydrous ether. This operation was repeated several times. The material was dried at 80°, at 15 mm. The composition of the substance was as follows:

Sample I.	5.185 mg. substance:	10.160 mg. CO ₂ and 3.575 mg. H ₂ O.
	8.225 " "	: 0.670 cc. N (29°, 760.7 mm.).
Sample II.	4.657 " "	: 9.010 mg. CO ₂ , 2.800 mg. H ₂ O, and
	0.191 mg. ash.	
Found.	Fraction I.	C 54.74, H 7.78, N 9.44
	Ash-free Fraction II.	" 55.75, " 7.10, ash 1.34

In the determination of amino nitrogen a purified sample was previously hydrolyzed for 20 hours with 20 per cent hydrochloric acid.

The total nitrogen and amino nitrogen were also determined on unhydrolyzed material.

Sample I.	7.425 mg. substance:	0.634 cc. N (26°, 760 mm.)	(Dumas).
	17.25 " "	:0.48 " " (20°, 761 ")	(Van Slyke).
Sample II.	6.620 " "	:0.558 " " (20°, 753 ")	(Dumas).
	20 " "	:0.54 " " (19°, 753 ")	(Van Slyke).

	Total N per cent	Total amino N per cent
Unhydrolyzed Fraction I.	9.41	1.60
" " II.	9.72	1.50
Hydrolyzed " II.		5.39

Preparation of Methyl Ester—10 gm. of the dried and finely powdered coloring matter were placed in a dark colored flask with 350 cc. of dry methyl alcohol containing 20 per cent hydrogen chloride and heated for 70 minutes with reflux in a water bath at 80°. The reaction product was thoroughly cooled with ice, diluted with ice water, and extracted with chloroform (five times) until the extract was only slightly colored. The chloroform extracts were washed first with a very dilute solution of sodium acetate (3 to 4 per cent) and then twice with water. The chloroform solution was dried with anhydrous sodium sulfate, concentrated under reduced pressure to a small volume, and precipitated with an excess of petroleic ether (30–40°).

It is essential that all these operations should be carried out with the exclusion of light, since the coloring matters are light-sensitive. This is readily accomplished by painting the outside of all flasks dark red. For analysis the material was dissolved in chloroform and reprecipitated with petroleic ether.

Sample I. 4.951 mg. substance:10.970 mg. CO₂, 2.922 mg. H₂O, 0.050 mg. ash.

5.605 mg. substance: 0.342 cc. N (23.5°, 7.57 mm.).

5.550 " " : 4.410 mg. AgI.

Found (ash-free). C 61.03, H 6.66, N 7.06, OCH₃ 10.59, ash 1.00

Sample II. 4.541 mg. substance:10.186 mg. CO₂ and 2.895 mg. H₂O.

8.360 " " : 0.555 cc. N (29°, 760 mm.).

Found (ash-free). C 61.32, H 7.14, N 7.55, ash 0.26

After hydrolysis of the ester with 20 per cent hydrochloric acid the amino nitrogen content was still 3.2 per cent. The ester was therefore treated once more with methyl alcoholic hydrogen chloride for 60 minutes at 80°. However, no important change in the percentage composition of the substance occurred.

The composition of the substance was as follows:

4.340 mg. substance: 9.700 mg. CO₂ and 2.537 mg. H₂O.

Found (ash-free). C 61.80, H 6.63, ash 1.39

Many attempts to obtain the ester in crystalline condition (e.g., from methyl alcohol, dilute ethyl alcohol, chloroform-petroleum ether, benzene-petroleum ether, etc.) were of no avail. Benzene proved to be the most suitable solvent for obtaining the material in a condition which seemed, under the microscope, to be of one definite form but which could not be said to be actually crystalline. The procedure was, however, attended with considerable loss of material.

Reduction of Ester with Hydriodic Acid—To a mixture of 30 cc. of hydriodic acid (sp. gr., 1.96) with 45 cc. of glacial acetic acid contained in a 150 cc. Claisen distilling flask were added 5 gm. of the ester and the solution was heated in a boiling water bath for 20 hours. The excess of free iodine was then removed with the necessary amount of phosphonium iodide, and the entire reaction mixture concentrated to a syrup under diminished pressure. The syrup was dissolved in dilute sodium carbonate and steam-distilled to remove all volatile matter. An oil having a disagreeable odor collected in the receiver.

The distillate was extracted with ether and the ether extract evaporated to dryness yielding an oil which gave a strongly positive reaction with Ehrlich's reagent. Attempts to crystallize the substance or to prepare a picrate were unsuccessful.

The brown-colored solution which remained in the flask after the steam distillation was acidified with dilute hydrochloric acid. A thick brown-colored precipitate was obtained which was filtered on a Buchner funnel and washed with water until free from mineral acid. As the organic acid was only slightly soluble in organic solvents, it was purified by dissolving in sodium carbonate solution and reprecipitating with dilute hydrochloric acid. The precipitate was washed free from hydrochloric acid by repeated centri-

fuging with water. This material was not entirely ash-free. Its composition was as follows:

4.120 mg. substance: 10.000 mg. CO_2 and 2.695 mg. H_2O .

5.438 " " : 0.240 cc. N (29° , 757 mm.).

Found (ash-free). C 67.87, H 7.49, N 5.08, ash 2.50

Esterification with Methanol of the Product Obtained by Hydriodic Acid Reduction—The acid was covered with a layer of absolute methyl alcohol, saturated with hydrogen chloride, and allowed to stand overnight. The product was then poured on to ice, diluted with water, and extracted with chloroform. The chloroform extract was washed with water, dried with anhydrous sodium sulfate, and concentrated to a small volume under diminished pressure. Precipitation with petroleic ether yielded the ester as a brown fluffy powder. For analysis the ester was purified by dissolving in chloroform, removing all traces of unesterified acid by shaking with dilute sodium carbonate solution, and reprecipitating with petroleic ether. The composition of the substance was as follows:

Sample I. 4.285 mg. substance: 10.080 mg. CO_2 and 2.630 mg. H_2O

5.873 " " : 0.249 cc. N (25° , 745 mm.).

4.977 " " : 3.665 mg. AgI.

Sample II. 6.820 " " : 0.285 cc. N (24° , 750 mm.).

" III. 3.895 " " : 9.415 mg. CO_2 , 2.495 mg. H_2O , and 0.065 mg. ash.

10.305 mg. substance: 0.371 cc. N (28° , 761 mm.).

$\text{C}_{17}\text{H}_{23}\text{O}_4\text{N}_1$. Calculated. C 66.84, H 7.60, N 4.60, OCH_3 10.17

Sample	I	} Ash-	{	"	65.66,	"	6.97,	"	4.76,	"	9.72,	ash 1.70
Found	"			II	"	67.02,	"	7.28,	"	4.82	"	1.66
	"			III	free	"	67.02,	"	7.28,	"	4.12	"

Oxidation of Ester with Chromic Acid in Glacial Acetic Acid—5 gm. of the ester were dissolved in 150 cc. of glacial acetic acid and to this a solution of 9.5 gm. of chromic acid in 100 cc. of glacial acetic acid were added in one portion. A heavy precipitate settled out at once. The mixture was warmed on a boiling water bath for 1 hour, after which time the precipitate had completely disappeared and the color of the solution turned green. The reaction product was concentrated under diminished pressure to complete dryness. The residue was dissolved in 20 per cent sulfuric acid and extracted eight to ten times with ether. The ether solution was

washed with sodium carbonate solution and water and dried with anhydrous sodium sulfate. After removal of the solvent, a small quantity of oxidation product remained. The products of six similar experiments were combined and the oil distilled *in vacuo* (boiling point 180–200°, p = 11 mm.). It was redistilled for analysis. The yield of the 30.0 gm. was about 2.0 gm. The composition of the substance was as follows:

5.370 mg. substance:	11.010 mg. CO ₂ and 3.000 mg. H ₂ O.
10.175 " " :	0.527 cc. N (30°, 760 mm.).
C ₉ H ₁₁ O ₄ N. Calculated.	C 54.82, H 5.63, N 7.11
C ₁₀ H ₁₃ O ₄ N. " "	" 56.87, " 6.16, " 6.64
Found.	" 55.91, " 6.25, " 5.83

A small amount of the distilled oxidation product was placed in a test-tube with a drawn out tip. The oil was dissolved in a small amount of ether. By slightly warming the tube, the ether was driven out and by immersing the tip in 5 per cent pure barium hydroxide solution and cooling the test-tube, it was completely filled to the neck without producing any cloudiness (absence of the formation of barium carbonate). The test-tube was then sealed and shaken overnight. A flocculent precipitate formed which was filtered off. The yield was just enough for an analysis. Determination of the barium identified it as a barium salt of the tribasic hematinic acid.

4.720 mg. substance:	3.975 mg. BaSO ₄ .
(C ₉ H ₉ O ₆) ₂ Ba ₃ . Calculated.	Ba 49.15. Found. Ba 49.56

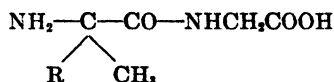
THE ACTION OF ACETIC ANHYDRIDE ON TERTIARY AMINO ACIDS AND DIPEPTIDES. ON CATALYTIC EFFECTS. THE HYDROLYSIS OF ACETYLDIPEPTIDES

BY P. A. LEVENE AND ROBERT E. STEIGER

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

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At the outset of this work the preparation of the series of dipeptides of the following type



was contemplated in order that a general study of their properties might be made. Preliminary experiments having indicated that the corresponding bromoacyl-glycines are aminated at an exceedingly low rate and that the isolation of the final products is most difficult, it seemed advisable to have recourse to the "azlactone method of synthesis" which was originated by Mohr¹ and which, in modified forms, has been employed lately with great success by Bergmann and his collaborators.² This entailed the preparation of the azlactones of the acetylated tertiary amino acids. While with *d,l*-phenylmethylaminoacetic acid no difficulties were encountered and the azlactone was always obtained in a good yield, the analogous compounds from α -aminoisobutyric acid and *d,l*-isovaline could not be isolated for reasons stated below. The

¹ Mohr, E., and collaborators, *J. prakt. Chem.*, **81**, 55, 71 (1910); **81**, 473 (1910); **81**, 333, 334 (1910).

² Bergmann, M., Stern, F., and Witte, C., *Ann. Chem.*, **449**, 277 (1926). Bergmann, M., and Zervas, L., *Z. physiol. Chem.*, **175**, 154 (1928). Bergmann, M., and Grafe, K., *Z. physiol. Chem.*, **187**, 196 (1930). See also Bergmann, M., Schmidt, V., and Miekeley, A., *Z. physiol. Chem.*, **187**, 264 (1930).

azlactone prepared was converted for identification purposes into N-acetyl-*d,l*-phenylmethylaminoacetic acid and its amide. The first of these derivatives had already been obtained, though in not as pure a state, by the action of an excess of acetic anhydride on the amino acid in the presence of pyridine.³ The pure acetyl-amino acid melts at 202–203° (corrected).

When α -aminoisobutyric acid (0.1 mol) was boiled with an excess of acetic anhydride (100 cc.) deemed sufficient to bring about complete transformation of the amino acid into the azlactone, only solid products, partly insoluble and partly soluble in the excess of acetic anhydride, were obtained (Experiment 1). The total yield of these crude products was very large; since but little amino acid remained to be accounted for, only a small amount of the azlactone formed could possibly have remained and been lost in distillation.⁴ The purified solids appear to be complex condensation products. They are probably mixtures of substances of rather high molecular weight. On the other hand, in the absence of such catalysts as traces of chlorine and ammonia commonly present in the commercially available amino acids, and of boiling stones such as were added to the reaction mixtures in the initial experiments, substances of this type are not formed and the reaction takes a fairly normal course (see Table I).

With pure α -aminoisobutyric acid a clear solution was obtained after short boiling. No insoluble materials were formed later, not even when a boiling stone was added to the solution. The main product of this operation, the volatile azlactone, was however lost when the acetic acid and the excess of acetic anhydride were removed by distillation. There remained an oily residue which crystallized spontaneously after removal by means of xylene of the last traces of acetic anhydride and of azlactone. The crystals (yield, 20 per cent of the theory) were those of a compound $C_{10}H_{16}O_3N_2$ (Experiment 3). For a summary of these experiments, see Table I.

³ Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, **79**, 102 (1928).

⁴ Some time later, a short passage in Mohr, E., and Geis, T., *J. prakt. Chem.*, **81**, 56 (1910), referring specifically to the matter, came to our attention. Mohr states that in an experiment with 2 mols of acetic anhydride probably little azlactone was lost and that the bulk of the α -aminoisobutyric acid remained in the residue from the distillation in the form of an unidentified material.

An experiment similar to the foregoing was then carried out with pure anhydrous *d,l*-isovaline (Experiment 1). The course of the reaction, however, was as far as could be seen, not modified by the addition of a small amount of ammonium chloride and of a boiling stone to the mixture of this amino acid with acetic anhydride (Experiment 2).

Observations made at a later time in the course of this work may be mentioned here. The dipeptide α -aminoisobutyryl- α -ami-

TABLE I

Action of Acetic Anhydride on α -Aminoisobutyric Acid

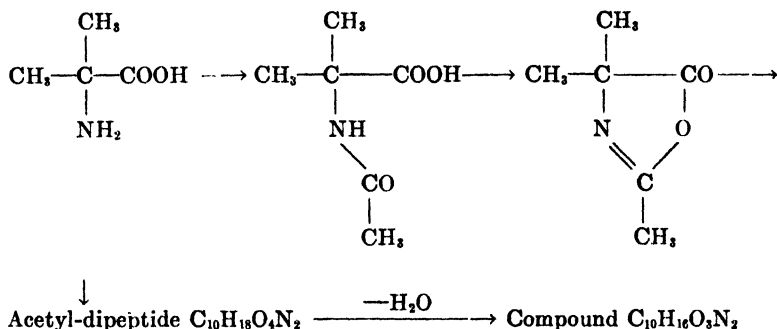
Weights of the solid products obtained in Experiments 1, 2, and 3 with 10.4 gm. (0.1 mol) of α -aminoisobutyric acid and 100 cc. of 95 per cent acetic anhydride.

Experiment No.	Nature of amino acid used	Boiling stone added	In acetic anhydride		Nature of Residue B
			Insoluble fractions (Residue A) washed	Soluble products, crude (Residue B)	
1	Kahlbaum's. Contained traces of Cl and NH_3	At very start	0.65 gm. (No. 284)	7.25 gm. (Residue B_1)	Complex mixture
2	Purified, free of traces of Cl and NH_3	At very start	0.39 gm. (No. 287)	2.85 gm. (Residue B_2)	Lactone $\text{C}_{10}\text{H}_{16}\text{O}_2\text{N}_2$ and another material
3	Same as for Experiment 2	30 min. after start	0.00 gm.	2.08 gm.	Lactone $\text{C}_{10}\text{H}_{16}\text{O}_2\text{N}_2$

noisobutyric acid undergoes rather readily dehydration in a medium of hot glacial acetic acid. The rate of this reaction seems to be largely determined by the amount of impurities, that is, of traces of ammonia, contained in the dipeptide used. A sample of a high degree of purity (no coloration and no cloudiness with Nessler's reagent) gave a clear solution with this solvent, whereas a slightly less pure material (which showed but a slight cloudiness but no coloration on long standing with Nessler's reagent) gave,

when dissolved in hot acetic acid, a strongly opalescent solution. In both cases there was α -aminoisobutyric acid anhydride formed. Because of lack of material, quantitative experiments under conditions strictly comparable could not be carried out.

The steps leading to the formation of the compound $C_{10}H_{16}O_3N_2$ from α -aminoisobutyric acid are the following.



A certain amount of azlactone of N-acetyl- α -aminoisobutyric acid formed reacts with an equivalent quantity of unchanged amino acid (acetylation takes place slowly⁵) to give an acetyl-dipeptide (broad sense designation) which by loss of water in one of various possible ways is then converted into the final product.

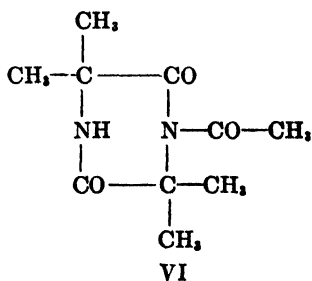
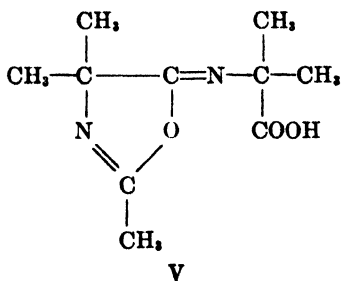
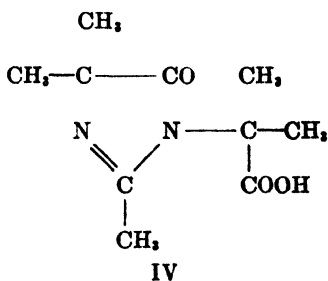
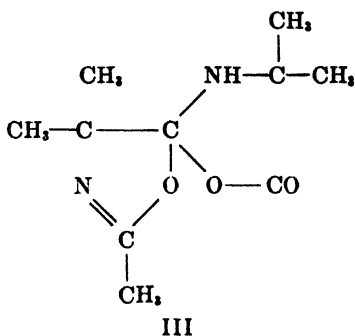
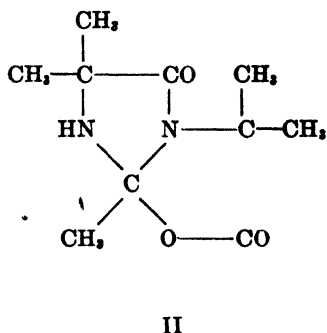
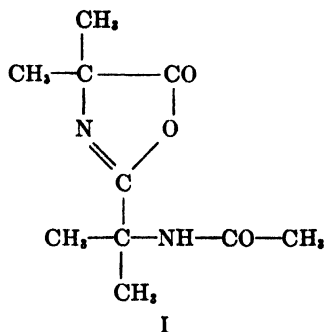
In Table II are given the structural formulæ of the cyclic compounds $C_{10}H_{16}O_3N_2$ that can be derived from N-acetyl- α -aminoisobutyryl- α -aminoisobutyric acid. Inasmuch as the isolated substance behaves on titration as would a typical lactone, but one of the first three of these structures comes into consideration for this compound. The first (and the only one hitherto known) lactone of an N-acyl-dipeptide has been prepared by Mohr and Stroschein.⁶ N-Benzoyl-*d,l*-alanyl- α -aminoisobutyric acid when heated with acetic anhydride to 100° underwent dehydration. Mohr ascribed to his substance a structure similar to that of Compound I (see Table II) but no evidence to that effect was produced. Its behavior towards water was not investigated.

⁵ Quite some time elapses before even pure α -aminoisobutyric acid (0.1 mol) is completely dissolved in the chosen amount of acetic anhydride (100 cc.). *d,l*-Isovaline and *d,l*-phenylmethylaminoacetic acid are dissolved much more rapidly under identical conditions.

⁶ Mohr, E., and Stroschein, F., *J. prakt. Chem.*, **81**, 478, 499 (1910).

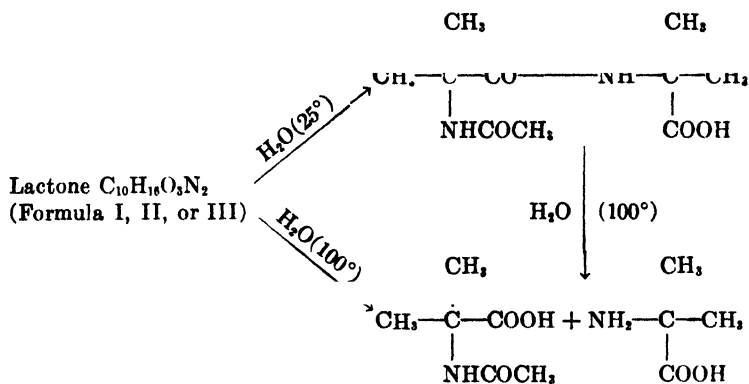
The lactone $C_{10}H_{16}O_3N_2$ is hydrolyzed by water at room temperature and gives almost quantitatively N-acetyl- α -aminoisobutyryl- α -aminoisobutyric acid.

TABLE II
Compounds $C_{10}H_{16}O_3N_2$



butyryl- α -aminoisobutyric acid. This substance was also prepared in a different way, that is, by acetylation of the corresponding

dipeptide under conditions described in the experimental part. The lactone is decomposed by water at 100°. The solution contains an equimolecular mixture of α -aminoisobutyric acid and of N-acetyl- α -aminoisobutyric acid. These substances were actually isolated. The nature of the latter was established by a direct comparison with the compound obtained on acetylation of α -aminoisobutyric acid with 1.1 mols of acetic anhydride in acetic acid solution. This substance had not yet been prepared.



The very formation of these scission products and the ease with which this reaction takes place are of distinct significance. But how premature any speculation at this stage of the work as to the probable structure of the lactone $C_{10}H_{16}O_3N_2$ would have been is shown by the following. N-Acetyl- α -aminoisobutyryl- α -aminoisobutyric acid was not only found to be as unstable towards boiling water as the lactone itself but also to give the same products of scission. This last finding was wholly unexpected. It is known that the acetyl derivatives of dipeptides when boiled for a moderate length of time with dilute mineral acids undergo deacetylation and yield the acid salts of the dipeptides. Also it had been previously established that dipeptides, such as glycyl- α -aminoisobutyric acid, containing a tertiary amino acid as second component, were, unlike the peptides wholly composed of ordinary amino acids, very resistant towards hydrolytic agents (erepsin and alkali).⁷

⁷ Levene, P. A., Steiger, R. E., and Bass, L. W., *J. Biol. Chem.*, **82**, 156, 164 (1929). Levene, P. A., Bass, L. W., and Steiger, R. E., *J. Biol. Chem.*, **82**, 168, 169 (1929).

α -Aminoisobutyryl- α -aminoisobutyric acid was then prepared. It was found to be absolutely resistant to the action of boiling water (4 hours). Thus, with the acetyl derivative of this dipeptide, a sharply marked case of substitutional activation has been revealed.⁸ As is readily surmised, the nature of the two amino acid components of the system determines the instability of the peptidic linkage, the very degree of this instability depending largely upon the structure of the first amino acid. This was established in the following way. N-Acetyl-glycyl- α -aminoisobutyric acid was prepared. This is also hydrolyzed by boiling water but only to the extent of 10 per cent in the course of 4 hours (against 90 per cent as found in the case of the former compound). Whether deacetylation takes place or whether the peptide linkage is severed was not determined. N-Acetyl-*d*,*l*- α -aminophenylmethylacetyl- α -aminoisobutyric acid was then prepared by condensation of the azlactone of N-acetyl-*d*,*l*-phenylmethylaminoacetic acid with α -aminoisobutyric acid. This acetyl-dipeptide could not be obtained by the method seemingly general for reactions of this kind proposed by Mohr and Stroschein⁹ and adopted by Bergmann and his collaborators²; namely, by condensing the components in alkaline acetone-water mixtures. The azlactone was invariably hydrolyzed to the corresponding acetyl-amino acid and no other reaction took place. The acetyl-dipeptide was, however, very readily formed when the azlactone was added to a hot suspension of the amino acid (used in slight excess) in glacial acetic acid. This procedure is similar to Knoop and Blanco's method¹⁰ of acetylating amino acids by means of acetic anhydride. It is believed that it will prove valuable not only in the case of tertiary amino acids but also in the more general instance of most other amino acids as well as in the case of peptides.

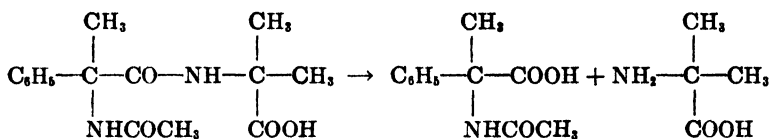
This last acetyl-dipeptide is even more readily decomposed by boiling water than the one discussed first. Hydrolysis to the

⁸ So far as could be ascertained, no record is to be found in the literature of any attempt to hydrolyze acetyl-dipeptides at the pH and temperature of their boiling aqueous solutions. The extent of the hydrolysis increases definitely with the concentration of the solution.

⁹ Mohr, E., and Stroschein, F., *J. prakt. Chem.*, **82**, 334 (1910).

¹⁰ Knoop, F., and Blanco, J. G., *Z. physiol. Chem.*, **146**, 273 (1925).

extent of 94 per cent was observed in the course of 4 hours. The products of scission were isolated. The reaction is as follows:



These findings on the instability of substituted dipeptides containing a tertiary amino acid as first component afford a ready interpretation of certain observations made by Mohr and Geis¹¹ as long ago as 1910. These authors had prepared N-benzoyl- α -aminoisobutyryl-glycine by fusing the azlactone of N-benzoyl- α -aminoisobutyric acid with glycine. The reaction product was recrystallized several times from boiling water. It yielded a mixture of crystals which softened at 175° before melting at 191° and were soluble in about 13.8 parts of boiling water. The molecular weight determined by titration was only 254.4 instead of 264.15. This material undoubtedly contained about 17 per cent of N-benzoyl- α -aminoisobutyric acid (this softens at 195°, melts at 198°, dissolves in 28 parts of boiling water, and has a molecular weight of 207.11) formed by partial decomposition of the original substance in boiling water.

EXPERIMENTAL

Action of Acetic Anhydride on α -Aminoisobutyric Acid

Experiment 1—100 cc. of 95 per cent acetic anhydride (about 0.5 mol) were added to 10.4 gm. (0.1 mol) of finely powdered α -aminoisobutyric acid (Kahlbaum's) containing traces of ammonium chloride. A boiling stone was added. The 200 cc. Pyrex round bottom flask was equipped with a reflux condenser and immersed in an oil bath kept at 150°. Boiling took place after 8 minutes. No clear solution was obtained at any time. The suspension was kept at boiling temperature for $\frac{1}{2}$ hour. It was then rapidly cooled down to room temperature. It contained an appreciable amount of insoluble material. This was filtered off with suction. It was thoroughly washed with xylene (Residue A₁).

¹¹ Mohr, E., and Geis, T., *J. prakt. Chem.*, **81**, 71 (1910).

Residue A₁ was well washed with water and then with absolute alcohol. It weighed dry, 0.65 gm. When heated to 250° this material did not melt.

No. 284.

4.610 mg. substance: 9.360 mg. CO₂ and 3.205 mg. H₂O.

5.845 " " : 0.749 cc. N (27°, 762.5 mm.) (Dumas).

5.125 " " : 10.395 mg. CO₂ and 3.700 mg. H₂O.

6.360 " " : 0.800 cc. N (23.5°, 764.7 mm.) (Dumas).

Found. C 55.36, 55.31; H 7.77, 8.07; N 14.62, 14.53

The filtrate of Residue A₁ was concentrated under reduced pressure. There remained an oily red residue. It was dissolved in xylene, which was then evaporated off. This operation was repeated several times in order to remove any remaining acetic anhydride and some other volatile material. The temperature of the bath was finally raised to 90°. The flask with its contents was then kept in a vacuum desiccator over phosphorus pentoxide, soda-lime, and paraffin chips. The residue became solid after some time. It weighed 7.25 gm. (Residue B₁). It was readily soluble in hot benzene but no deposit was formed on cooling nor was a crystalline precipitate obtained when other solvents were added to this solution. The remainder of Residue B₁ was boiled for several hours with water. The filtrate containing some water-soluble substances was discarded. This product was dissolved in boiling absolute alcohol; the solution was clarified with norit and concentrated to dryness under reduced pressure. The residue was suspended in water, filtered off, washed with water, and dried. When heated to 250° it did not melt. It was soluble in 0.1 N NaOH; insoluble, however, in 0.1 N HCl.

No. 302.

4.800 mg. substance: 9.690 mg. CO₂ and 3.485 mg. H₂O.

6.965 " " : 0.879 cc. N (24°, 769.2 mm.) (Dumas).

Found. C 55.05, H 8.12, N 14.38 (Dumas)

" O (Van Slyke)

0.1600 gm. substance: 2.50 cc. 0.1 N NaOH (titration, phenolphthalein).

Acidity of 100 gm., in cc. of 1.0 N NaOH, 156.3.

A small amount of a material (No. 303) identical with this one was obtained in very much the same manner from the reaction product (Residue B₂) of the following experiment.

Experiment 2—The reaction was carried out in the same way as in Experiment 1. However, pure α -aminoisobutyric acid, free of traces of chlorine and ammonia, was used. A boiling stone consisting of a fragment of a porous porcelain plate was added to the mixture from the start. No clear solution was obtained at any time. The insoluble material was separated by filtration from the supernatant liquid. It was washed with xylene (Residue A₂).

Residue A₂ was thoroughly washed with water and then with absolute alcohol. When dry, it weighed 0.39 gm. Its composition was then that of a compound C₁₇H₃₀O₅N₄ or C₃₄H₆₀O₁₀N₈ (C 55.10, H 8.16, N 15.14). However, whether this material was pure or was but a mixture of substances could not be determined. When heated to 250°, it did not melt. It was slightly, but entirely, soluble in pyridine.

No. 287.

5.200 mg. substance: 10.500 mg. CO₂ and 3.815 mg. H₂O.

6.790 " " : 0.916 cc. N (30°, 755.3 mm.) (Dumas).

Found. C 55.06, H 8.20, N 15.09

The filtrate of Residue A₂ was concentrated under reduced pressure. The acetic anhydride remaining in the residue was removed by means of xylene in the manner indicated before. The product of the reaction crystallized in the course of the last evaporation. The bath was then at 70°. Some oil (the α -lactone of N-acetyl- α -aminoisobutyric acid) was given off when the temperature of the bath was raised to 85–90°. The crystalline residue was dried in a vacuum desiccator over phosphorus pentoxide, soda-lime, and paraffin chips. It weighed 2.85 gm. (Residue B₂). This product was dissolved in hot benzene. The crystals deposited on cooling were in the main those of a lactone C₁₀H₁₆O₃N₂ but their separation from those of an accompanying impurity of more complex nature could not be accomplished by fractional crystallization. The mixture was therefore treated with water at 20–25°, wherein the lactone is readily soluble. There remained an insoluble fraction which was filtered off (the filtrate yielded, on slow concentration, crystals of N-acetyl- α -aminoisobutyryl- α -aminoisobutyric acid (see that paragraph)). It was thoroughly washed with water and dried. When heated to 250°, it did not melt.

No. 303.

4.020 mg. substance: 8.080 mg. CO_2 and 3.025 mg. H_2O .

6.360 " " : 0.793 cc. N (25.5°, 758.3 mm.) (Dumas).

Found. C 54.81, H 8.42, N 14.21

0.1390 gm. substance: 2.16 cc. 0.1 N NaOH (titration, phenolphthalein).

Acidity of 100 gm., in cc. of 1.0 N NaOH, 155.4.

Experiment 3. Preparation of Lactone $\text{C}_{10}\text{H}_{16}\text{O}_3\text{N}_2$ —100 cc. of 95 per cent acetic anhydride (about 0.5 mol) were added to 10.4 gm. (0.1 mol) of finely powdered pure α -aminoisobutyric acid, free of traces of chlorine and ammonia. (This amino acid was not in any way different from that employed in Experiment 2. Both samples were taken from the same batch.) The 200 cc. round bottom Pyrex flask was equipped with a reflux condenser and was immersed in an oil bath kept at 150°. The mixture started to boil after 6 minutes. After 4 minutes of effective boiling all the amino acid had dissolved. A clear solution was obtained. Only some time after this was a boiling stone added to the mixture. The solution was kept at boiling temperature for a total of 30 minutes. As it remained perfectly clear during all this time and even after cooling to room temperature, it was directly concentrated under reduced pressure. The oily residue was repeatedly taken up in xylene, this being evaporated off as often in between times. The acetic anhydride and some α -lactone of N-acetyl- α -aminoisobutyric acid thus being removed, the residue crystallized spontaneously in the course of the last evaporation. The flask with its contents was kept in a vacuum desiccator over phosphorus pentoxide, soda-lime, and paraffin chips. The crystals weighed 2.08 gm. They were those of a lactone $\text{C}_{10}\text{H}_{16}\text{O}_3\text{N}_2$.

Lactone $\text{C}_{10}\text{H}_{16}\text{O}_3\text{N}_2$

It can be easily prepared by the action of acetic anhydride on pure α -aminoisobutyric acid. The method of preparation is given above under Experiment 3; the conditions must be rigorously observed. The crude material obtained (yield, 20 per cent of the theory) was recrystallized from hot benzene. The crystals obtained were constantly kept stored over phosphorus pentoxide. They melted at 143–144° (corrected) with slight preliminary sintering. For analysis the substance was dried at 100° under

reduced pressure. It then sublimed in part, but no alteration took place.

No. 311.

4.845 mg. substance: 10.045 mg. CO_2 and 3.064 mg. H_2O .

6.087 " " : 6.96 cc. N (24.5° , 755.6 mm.) (Dumas).

$\text{C}_{10}\text{H}_{16}\text{O}_3\text{N}_2$ (212.15). Calculated. C 56.56, H 7.60, N 13.21

Found. " 56.53, " 7.07, " 13.05

A freshly prepared solution of the lactone in water (in which the substance is readily soluble) contained no amino nitrogen nor was any detectable at any time on keeping. Solutions of the lactone in 2 mols of 0.1 N HCl and of 0.1 N NaOH were kept for 24 hours at 25° and amino nitrogen determinations were made. As in the case with pure water, no amino nitrogen was found. The lactone is hydrolyzed by water at room temperature to N-acetyl- α -aminoisobutyryl- α -aminoisobutyric acid and decomposed by water at 100° to N-acetyl- α -aminoisobutyric acid and α -aminoisobutyric acid. The lactone seems to be stable in absolute alcohol at room temperature.

Hydrolysis of Lactone $\text{C}_{10}\text{H}_{16}\text{O}_3\text{N}_2$ with Water at 25°

The lactone $\text{C}_{10}\text{H}_{16}\text{O}_3\text{N}_2$ is hydrolyzed by water at room temperature. Ring opening takes place; ultimately N-acetyl- α -aminoisobutyryl- α -aminoisobutyric acid (see that paragraph) is formed. Little can be said about the rate of formation of this derivative. A freshly prepared solution of the lactone in water is distinctly acid towards acid indicators. When 0.1 N NaOH is added gradually to such a solution, phenolphthalein being used as an indicator, the alkali is very rapidly consumed up to a certain stage in the experiment, after which the pink coloration is more persistent. When the end-point is practically reached, it will only disappear in the course of several hours. A permanent coloration was attained with the following.

0.1061 gm. (0.0005 mol) lactone: 5.01 cc. 0.1 N NaOH.

Calculated for 0.0005 mol R·COOH: 5.00 cc. 0.1 N NaOH.

No amino nitrogen was detected in this solution.

Hydrolysis of Lactone $C_{10}H_{16}O_3N_2$ with Water at 100° , and Isolation of the Products of Hydrolysis

About 80 cc. of boiling water were added to 0.4243 gm. (0.002 mol) of the pure lactone $C_{10}H_{16}O_3N_2$ (No. 311) contained in a 100 cc. volumetric flask. The clear solution was kept at boiling temperature for 4 hours. Water was then added to bring its volume to 100 cc. No attention was paid to a slight cloudiness formed and the following determinations were made on this solution (No. 312).

4.00 cc. gave 1.74 cc. N at 21° and 761 mm. (Van Slyke, 45 minutes).¹²
 10.00 " required 4.20 cc. 0.1 N HCl (Kjeldahl).
 10.00 " consumed 2.01 " 0.1 " NaOH (titration, phenolphthalein).

These results are to be interpreted as follows:

Calculated for a mixture of 0.002 mol of α -aminoisobutyric acid and 0.002 mol of N-acetyl- α -aminoisobutyric acid in 100 cc. of solution

$$\frac{NH_2 \text{ nitrogen}}{\text{Total nitrogen}} = 0.50. \quad \text{Acidity (on 100 cc.)} = 20.00 \text{ cc. } 0.1 \text{ N NaOH}^{13}$$

Found

$$\frac{NH_2 \text{ nitrogen}}{\text{Total nitrogen}} = 0.40. \quad \text{Titration (on 100 cc.)} = 20.10 \text{ cc. } 0.1 \text{ N NaOH}$$

The solution which remained after these determinations was clarified by filtration and then evaporated to dryness under reduced pressure. The crystalline residue was kept in a vacuum desiccator over phosphorus pentoxide until completely dry. It was then rapidly extracted with a small amount of absolute alcohol.

The insoluble fraction was washed with absolute alcohol (the washings were discarded) and dried. This material (No. 318) was

¹² The rate of deamination of tertiary aliphatic amino acids is considerably lower than that of ordinary amino acids. Correct amino nitrogen figures, with Van Slyke's apparatus, are obtained only after 45 minutes of shaking. See Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, **76**, 302, 318 (1928).

¹³ α -Aminoisobutyric acid does not consume any alkali, phenolphthalein being used as an indicator.

found to contain 13.71 per cent of total nitrogen (Kjeldahl) and 13.33 per cent of amino nitrogen (Van Slyke). A further and larger amount of the same material had been isolated previously from the residue of another hydrolysis experiment similar to the foregoing. This residue was dissolved in a hot mixture of water and alcohol. The clear solution on cooling in ice and water yielded a microcrystalline precipitate which was insoluble in cold alcohol. It was dried at 90°. The analysis showed this to be α -amino-isobutyric acid.

No. 288.

5.880 mg. substance: 10.080 mg. CO₂ and 4.560 mg. H₂O.

5.195 " " : 0.606 cc. N (23°, 759.7 mm.) (Dumas).

10.000 " " : 2.37 " " (23°, 762.1 " (Van Slyke).¹²

C₄H₉O₂N (103.08). Calculated. C 46.57, H 8.80, N 13.59

Found. " 46.74, " 8.67, " 13.44 (Dumas)

" 13.33 (Van Slyke)

The alcoholic extract (see above) was evaporated to dryness. The residue was completely dried over phosphorus pentoxide and was dissolved in boiling ethyl acetate. The crystals deposited on cooling melted at 192–193° (uncorrected) and at 194–195° (corrected). The substance thus isolated was *N*-acetyl- α -aminoisobutyric acid for a mixture with the somewhat purer substance obtained on direct acetylation of α -aminoisobutyric acid (see the following paragraph) melted at the same temperature.

N-Acetyl- α -Aminoisobutyric Acid

7.2 gm. of 95 per cent acetic anhydride (0.066 mol) were added to a warm mixture of 6.2 gm. (0.06 mol) of α -aminoisobutyric acid and 60 gm. of glacial acetic acid.¹⁴ This mixture was boiled for a short time; a clear solution resulted. It was allowed to cool. It was then concentrated to a small volume under reduced pressure. The acetyl derivative crystallized. Toluene was added to the mixture and then was evaporated off, this in order to remove all the acetic acid present. The dry residue was suspended in a mixture of toluene and heptane (b.p. 90–100°). It was filtered off and washed with heptane. The air-dried material weighed 9.30

¹⁴ The general method devised by Knoop and Blanco (Knoop, F., and Blanco, J. G., *Z. physiol. Chem.*, **146**, 273 (1925)) was used.

gm. (theory, 8.71 gm.). It contained some unchanged amino acid. The complete separation was effected as follows: The crude material was extracted with hot absolute alcohol. The insoluble fraction was discarded and the filtrate was concentrated to dryness. This residue was almost entirely soluble in hot absolute alcohol. The solution was clarified with norit and concentrated to a small volume under reduced pressure. Crystallization took place. The crystals were washed with a small amount of cold absolute alcohol and then more freely with ethyl acetate. They were dissolved in boiling ethyl acetate. An insoluble fraction was removed by filtration and discarded. Abundant crystallization took place on cooling. The analysis of this substance was as follows:

No. 319.

4.970 mg. substance: 9.085 mg. CO₂ and 3.260 mg. H₂O.

5.198 " " : 0.437 cc. N (25°, 754.5 mm.) (Dumas).

C₆H₁₁O₃N (145.10). Calculated. C 49.62, H 7.64, N 9.66

Found. " 49.99, " 7.34, " 9.56 (Dumas)

" 0 (Van Slyke)

0.1451 gm. substance: 9.99 cc. 0.1 N NaOH (titration, phenolphthalein).

Calculated for 0.001 mol: 10.00 cc. 0.1 N NaOH.

This compound is quite soluble in water and in alcohol, but much less so in hot ethyl acetate. It melted at 193–194° (uncorrected) and at 195–196° (corrected) with preliminary sintering.

α-Aminoisobutyryl-α-Aminoisobutyric Acid

This dipeptide has already been obtained by amination for 3 days of α-bromoisobutyryl-α-aminoisobutyric acid¹⁵ with 5 parts of 25 per cent ammonium hydroxide at 37°. A modification of this procedure and some information regarding the stability of this dipeptide are given in the following. A mixture of 1 part of crude bromo compound and 10 parts by weight of ammonium hydroxide of sp. gr. 0.90 was kept for 10 days at 41° ± 2°. The dry residue of this solution still contained a large amount of untransformed bromo compound in the form, however, of the stable ammonium salt. It is best treated in the cold with a slight excess of dilute hydrochloric acid which decomposes this salt with precip-

¹⁵ Abderhalden, E., and Gebelein, F., *Z. physiol. Chem.*, **152**, 129 (1926).

itation of the free acid and dissolves the dipeptide. The filtrate containing the latter was then freed of halogen ions in the usual way and concentrated. The dipeptide was precipitated with alcohol. Yield, over 24 per cent (rather large losses of material were sustained before the method by extraction with acid was adopted). The product obtained contained traces of ammonia. It was recrystallized twice from water and alcohol. The final product gave no coloration with Nessler's reagent nor did the mixture become cloudy on long standing. It dissolved in hot glacial acetic acid to give a clear solution. The analysis was as follows:

No. 349.

4.539 mg. substance: 8.520 mg. CO₂ and 3.419 mg. H₂O.

4.340 " " : 0.557 cc. N (27°, 761 mm.) (Dumas).

C₈H₁₆O₂N₂ (188.15). Calculated. C 51.02, H 8.57, N 14.89

Found. " 51.18, " 8.42, " 14.61

Moisture, 5.69 per cent

The mother liquors of crystallization of the preceding were combined. They yielded a less pure material (Sample 2). It gave no coloration with Nessler's reagent but the solution became cloudy on long standing. Its solution in hot glacial acetic acid was strongly opalescent.

This dipeptide is not hydrolyzed by boiling water. A solution of 0.1822 gm. of dipeptide in about 20 cc. of water was boiled for 4 hours under a reflux. Its volume was then brought to 25 cc. The following determinations were made on this solution (No. 351).

2.00 cc. gave 1.95 cc. N at 23° and 752 mm. (Van Slyke, 45 minutes).¹²

10.00 " required 7.89 cc. 0.1 N HCl (Kjeldahl).

Hence the amino nitrogen:total nitrogen ratio found was 0.49 against 0.50 as calculated and 0.51 as found for the blank.

α-Aminoisobutyric Acid Anhydride

This compound is well known. It has been obtained by many authors in several various ways and more recently again by Abderhalden and Gebelein¹⁵ in the following manner. *α*-Aminoisobutyryl-*α*-aminoisobutyric acid was heated with diphenylamine until dehydration which set in at 180° was complete (200°). The observation has now been made that this dipeptide is rather

readily converted into its anhydride by hot glacial acetic acid and hence is always obtained in more or less large amounts as a by-product when acetylation of the dipeptide by means of acetic anhydride (used in small excess) is carried out in that solvent. The rate of dehydration seems to be largely determined by the amount of traces of ammonia present in the starting material used. An acetylation experiment performed on a highly pure sample (No. 349) is described in one of the following paragraphs. The yield of anhydride was low and that of acetyl-dipeptide good. A summary of a similar experiment with a slightly less pure material is given here. 1.88 gm. of moist dipeptide (Sample 2; 0.0094 mol, dry) gave with 10 cc. of glacial acetic acid, after some heating and shaking, a strongly opalescent solution. It deposited crystals of anhydride on cooling before the acetic anhydride could be and was added. The suspension was again brought to its boiling point and then was allowed to cool. It was diluted with water and evaporated to dryness. The residue was treated with sodium hydroxide until alkaline towards phenolphthalein. The anhydride was filtered off, washed with water, then with dilute acid, and again with water. It weighed dry 0.668 gm. (yield, 42 per cent of the theory). The filtrate and washings were discarded. This material was recrystallized from boiling acetic acid. The crystals were washed with acetic acid and with absolute alcohol. They weighed 0.44 gm.

No. 358.

4.020 mg. substance: 8.380 mg. CO_2 and 2.950 mg. H_2O .

4.268 " " : 0.618 cc. N (27° , 758 mm.) (Dumas).

$\text{C}_8\text{H}_{14}\text{O}_3\text{N}_2$ (170.13). Calculated. C 56.43, H 8.29, N 16.47

Found. " 56.84, " 8.21, " 16.43

N-Acetyl- α -Aminoisobutyryl- α -Aminoisobutyric Acid

1. *Preparation from the Lactone $\text{C}_{10}\text{H}_{16}\text{O}_3\text{N}_2$* —This substance can be easily prepared in a pure state. A dilute solution of the lactone $\text{C}_{10}\text{H}_{16}\text{O}_3\text{N}_2$ in water is kept at room temperature in a vacuum desiccator over phosphorus pentoxide until it is sufficiently concentrated. The acetyl-dipeptide separates in the form of large beautiful crystals. The yield is almost quantitative.

A fairly large amount of this compound was also obtained in this manner from the reaction product of Experiment 2 on α -ami-

noisobutyric acid. It proved to be as pure as the one prepared from the pure lactone of Experiment 3. The crystals were washed with cold water and dried over phosphorus pentoxide. For analysis they were dried at 62° (chloroform bath) under reduced pressure.

No. 306.

5.245 mg. substance: 10.015 mg. CO₂ and 3.650 mg. H₂O.

6.275 " " : 0.657 cc. N (24°, 776.7 mm.) (Dumas).

C₁₀H₁₈O₄N₂ (230.16). Calculated. C 52.14, H 7.88, N 12.17

Found. " 52.07, " 7.78, " 12.32 (Dumas)

" 0 (Van Slyke)

0.2302 gm. substance: 10.00 cc. 0.1 N NaOH (titration, phenolphthalein).

Calculated for 0.001 mol: 10.00 cc. 0.1 N NaOH.

The acetyl-dipeptide melts and decomposes between 223–225° (corrected). Some gas is evolved and a solid new substance is formed. Colorless prismatic needles are deposited in the upper part of the melting point tube.

2. *Preparation from α-Aminoisobutyryl-α-Aminoisobutyric Acid*—A suspension of 3.77 gm. of pure α-aminoisobutyryl-α-aminoisobutyric acid (No. 349; 0.0189 mol, dry) in 20 cc. of glacial acetic acid was gently heated and shaken until all the dipeptide had dissolved. 2.37 gm. of 95 per cent acetic anhydride (0.022 mol, 16 per cent excess) were added; the clear solution was brought to its boiling point and then allowed to cool gradually to room temperature. It was concentrated to dryness under reduced pressure. Crystallization set in at the beginning of this operation. The residue was treated with 1.0 N sodium hydroxide until the mixture was alkaline towards phenolphthalein. The suspension was filtered. There remained a residue of α-aminoisobutyric acid anhydride. It was thoroughly washed with water, then with dilute acid, and again with water. It weighed dry 0.574 gm. (yield, 18 per cent of the theory). The filtrate containing the sodium salt of the acetyl-dipeptide was acidified with 5.0 N HCl and was set aside for slow concentration in a vacuum desiccator over phosphorus pentoxide and soda-lime. After standing for some time, the deposit of crystalline acetyl-dipeptide which had formed was filtered off and well washed with cold water. The filtrate was not further concentrated but was discarded. The crystals weighed dry 1.81 gm. (yield, 42 per cent of the theory). They melted at

224–225° (corrected). An intimate mixture of this substance with that obtained by hydrolysis of the lactone melted at the same temperature.

3. *Hydrolysis with Water at 100°*—The acetyl-dipeptide is decomposed by water at 100°. A solution of 0.1151 gm. (0.0005 mol) of substance in about 20 cc. of water was boiled for 4 hours under a reflux. Its volume was then brought to 25 cc. The following determinations were made on this solution (No. 320).

4.00 cc. gave 1.83 cc. N at 23° and 763 mm. (Van Slyke, 45 minutes).¹²
10.00 " required 4.13 cc. 0.1 N HCl (Kjeldahl).

$$\frac{\text{NH}_3 \text{ nitrogen}}{\text{Total nitrogen}} = 0.45. \text{ Calculated. } 0.50$$

3.00 cc. consumed 0.60 cc. 0.1 N NaOH (titration, phenolphthalein).
Acidity (on 25 cc.): 5.00 cc. 0.1 N NaOH. Calculated. 5.00 cc.¹³

In another experiment a solution of 0.9207 gm. (0.004 mol) of acetyl-dipeptide in 160 cc. of water was boiled for 6 hours under a reflux. It was then evaporated under reduced pressure. The residue was dried and then extracted with hot ethyl acetate. N-Acetyl- α -aminoisobutyric acid was isolated from the filtrate and identified by a mixed melting point determination. The residue of α -aminoisobutyric acid (No. 364) was washed with ethyl acetate and absolute alcohol. It weighed after drying, 0.37 gm. (theory, 0.4124 gm., 0.004 mol).

0.2000 gm. required 19.20 cc. 0.1 N HCl (Kjeldahl).
Found. N 13.44. Calculated. N 13.59

Action of Acetic Anhydride on d,l-Isovaline

The following two experiments were initiated with the purpose of recording the behavior of an amino acid (isovaline), closely related to α -aminoisobutyric acid, under conditions similar to those specified above. As in the case of α -aminoisobutyric acid (Experiments 2 and 3), formation of azlactone undoubtedly takes place to a great extent. This material was similarly very volatile and hence was lost when the excess of acetic anhydride was distilled off. No attempts were made to isolate the remaining solid product of the reaction in a pure state and to establish its nature.

Experiment 1—The procedure was the same as that of Experiment 3 with α -aminoisobutyric acid. A mixture of 11.8 gm. (0.1 mol) of anhydrous *d,l*-isovaline,¹⁶ free of traces of chlorine and of ammonia, and 100 cc. of 95 per cent acetic anhydride (about 0.5 mol) was prepared. The flask was immersed in the oil bath kept at 150°. A short time afterwards, that is before the mixture had started to boil, the amino acid was completely dissolved. The solution was kept boiling for $\frac{1}{2}$ hour, during which time it remained perfectly clear. It was concentrated under reduced pressure. The oily reddish residue was freed in the usual way of acetic anhydride. It solidified only on being kept for a long time in a vacuum desiccator over phosphorus pentoxide, soda-lime, and paraffin chips. It weighed 3.75 gm.

Experiment 2—It was similar in every respect to the foregoing experiment. A small amount of ammonium chloride and a boiling stone, however, were added to the original mixture. These, as far as could be seen, had no effect on the reaction. The amino acid dissolved entirely in the acetic anhydride and no insoluble material was formed on boiling. The solid product of the reaction appeared to be the same as that obtained above.

Azlactone of N-Acetyl-d,l-Phenylmethylaminoacetic Acid

16.5 gm. (0.1 mol) of finely powdered *d,l*-phenylmethylaminoacetic acid dried at 100° (free of ammonium chloride)¹⁷ and 100 cc. of 95 per cent acetic anhydride (about 0.5 mol) were introduced into a 200 cc. round bottom Pyrex flask. The flask was equipped with a reflux condenser and immersed in an oil bath heated to 150°. The mixture began to boil after 10 minutes; the amino acid was completely dissolved after 3 more minutes. The mixture was kept for $\frac{1}{2}$ hour at boiling temperature. It was then concentrated under reduced pressure (water pump). There remained an oily residue. It was taken up in some xylene which was then evaporated off. This operation was repeated twice in order to remove completely any acetic anhydride. The final residue (21.2 gm.) was fractionated through a short Vigreux column sealed to a conical flask. The first fraction contained some xylene. It was in the main composed of pure azlactone. The main fraction, a

¹⁶ Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, **76**, 303 (1928).

¹⁷ Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, **76**, 308 (1928).

colorless oil distilling around 119°, under 6 mm. pressure, weighed 13.7 gm. (yield, 72 per cent of the theory). No attempts were made to convert it into a solid material. For identification purposes it was directly transformed into the corresponding acetyl-amino acid and its amide which are described below.

N-Acetyl-d, l-Phenylmethylaminoacetic Acid

A suspension of 13.7 gm. of the above described azlactone in 100 cc. of water was heated to a point where interaction took place rather suddenly. The hydrolysis product separated in the form of a hard beautifully crystalline mass. It was crushed in a mortar. It was thoroughly washed with water and dried. Yield, 13.2 gm. (84 per cent of the theory). Hydration takes place also on prolonged standing of the mixture at room temperature. The acetyl compound was recrystallized twice from hot 20 per cent by weight alcohol. It melted at 202-203° (corrected). The analysis was as follows:

No. 289.

5.825 mg. substance: 13.600 mg. CO₂ and 3.405 mg. H₂O.

6.460 " " : 0.377 cc. N (23°, 767 mm.) (Dumas).

C₁₁H₁₃O₃N (207.12). Calculated. C 63.73, H 6.33, N 6.76

Found. " 63.66, " 6.54, " 6.79

The same compound had been previously obtained,³ though not in as pure a state, by acetylation of the corresponding amino acid with an excess of acetic anhydride in the presence of pyridine. The product of that operation melted, as reported, at 192-193.5° (uncorrected). It probably contained, at that time, some volatile impurity for after an extensive period of drying, it was now found to melt at a much higher temperature, namely at 198° (uncorrected). A mixture of this material with the purer one now prepared melted at 200° (uncorrected).

N-Acetyl-d, l-α-Aminophenylmethylacetamide

The azlactone of N-acetyl-d, l-phenylmethylaminoacetic acid reacts very readily with ammonium hydroxide. It dissolves completely with evolution of heat in an excess of this reagent of sp. gr. 0.90. On cooling, crystallization of the amide takes place. This material was filtered off, washed with some water, dried,

and recrystallized from absolute alcohol. The analysis was as follows:

No. 278.

4.540 mg. substance: 10.685 mg. CO_2 and 2.825 mg. H_2O .

6.620 " " : 0.824 cc. N (31° , 754 mm.) (Dumas).

$\text{C}_{11}\text{H}_{14}\text{O}_2\text{N}_2$ (206.13). Calculated. C 64.04, H 6.85, N 13.59

Found. " 64.18, " 6.96, " 13.85

This product was then recrystallized twice from hot 20 per cent by weight alcohol and well dried. It melted at $191\text{--}192^\circ$ (corrected) with slight preliminary sintering.

N-Acetyl-d,l- α -Aminophenylmethylacetyl- α -Aminoisobutyric Acid

1. *Preparation*—10.0 gm. (0.0525 mol) of azlactone of N-acetyl-d,l-phenylmethylaminoacetic acid were added to a hot suspension of 6.20 gm. (0.06 mol, excess 0.0075 mol) of α -aminoisobutyric acid in 60 gm. of glacial acetic acid. The mixture was heated to its boiling point. A clear solution was obtained. It was allowed to cool gradually to room temperature. It was then evaporated to dryness under reduced pressure. The white, crystalline residue was suspended in toluene which was then evaporated off in order to remove all the acetic acid present. The residue was then shaken with water at room temperature. Practically all the acetyl-dipeptide remained undissolved. It was thoroughly washed with cold water which extracted some N-acetyl-d,l-phenylmethylaminoacetic acid (1.2 gm. were isolated from the combined filtrate and washings). It was then partly dried in a vacuum desiccator over phosphorus pentoxide, triturated and washed with ether, and then dried. Yield, 12.0 gm. (78 per cent of the theory). This material was dissolved in 17 parts by weight of a mixture of ethyl acetate (35 per cent) and glacial acetic acid (65 per cent by weight). The solution was filtered while hot. 12 parts by weight of ethyl acetate were added to the filtrate and this allowed to cool to room temperature. Crystallization takes place slowly and is complete only after several hours. The crystals were filtered off and washed with an ethyl acetate-acetic acid mixture containing 30 per cent by weight of acid. They retain a rather large amount of solvent which is given off on drying in a vacuum oven at 60° .

No. 359.

4.946 mg. substance: 11.150 mg. CO₂ and 2.960 mg. H₂O.

6.039 " " : 0.509 cc. N (28°, 760.7 mm.).

C₁₁H₂₀O₄N₂ (292.18). Calculated. C 61.61, H 6.90, N 9.59

Found. " 61.49, " 6.69, " 9.56

The acetyl-dipeptide melted sharply between 212–213° (uncorrected) and between 214–215° (corrected) with slight evolution of gas.

2. *Hydrolysis with Water at 100°*—160 cc. of water were added to 1.1685 gm. (0.004 mol) of acetyl-dipeptide and the suspension was boiled under a reflux. After half an hour, all the substance was dissolved. After 4 hours of effective boiling, amino and total nitrogen determinations were made on a portion of this solution (No. 362).

4.00 cc. gave 2.32 cc. N at 29° and 753 mm. (Van Slyke, 45 minutes).¹²

10.00 required 4.73 cc. 0.1 N HCl (Kjeldahl).

Hence, the amino nitrogen:total nitrogen ratio was 0.47 and the substance had suffered hydrolysis to the extent of 94 per cent. The remainder of the solution was kept for 2 more hours at boiling temperature. It was evaporated under reduced pressure. The residue was dried and then extracted with hot ethyl acetate. The filtrate deposited crystals of N-acetyl-phenylmethylaminoacetic acid on cooling. This substance was identified by a mixed melting point determination. The fraction insoluble in ethyl acetate was α -aminoisobutyric acid.

N-Acetyl-Glycyl- α -Aminoisobutyric Acid

1. *Preparation*—10 cc. of glacial acetic acid were added to 1.60 gm. (0.01 mol) of dry glycyl- α -aminoisobutyric acid.¹³ The dipeptide dissolved at room temperature on shaking. 1.19 gm. of 95 per cent acetic anhydride (0.011 mol) were added, the solution was brought to its boiling point, and then allowed to cool gradually to room temperature. It was diluted with toluene and then concentrated to dryness under reduced pressure. Crystallization set in at the very beginning of the distillation. Toluene was again added and then evaporated off in order to remove all

¹² Levene, P. A., Steiger, R. E., and Bass, L. W., *J. Biol. Chem.*, **82**, 159 (1929).

the acetic acid. The residue was entirely soluble in hot acetone. The filtrate was concentrated to a small volume, the crystals then deposited were filtered off and washed with acetone. They weighed 0.58 gm. Another fraction was isolated from the mother liquors of crystallization by precipitation with ether. It weighed 0.71 gm. Total, 1.29 gm. (theory, 2.02 gm.). The analysis of the first fraction was as follows:

No. 355.

4.811 mg. substance: 8.355 mg. CO_2 and 3.115 mg. H_2O .

4.744 " " : 0.584 cc. N (27° , 753.5 mm.) (Dumas).

$\text{C}_8\text{H}_{14}\text{O}_4\text{N}_2$ (202.13). Calculated. C 47.50, H 6.98, N 13.86

Found. " 47.35, " 7.24, " 13.88

Each fraction of the acetyl-dipeptide softened at 188° and melted between $189\text{--}190^\circ$ (uncorrected) or between $190\text{--}191^\circ$ (corrected).

2. *Hydrolysis with Water at 100°* —A solution of 0.1011 gm. (0.0005 mol) of substance in about 20 cc. of water was boiled for 4 hours under a reflux. Its volume was then brought to 25 cc. The following determinations were made on this solution (No. 357).

4.00 cc. gave 0.23 cc. N at 28° and 763 mm. (Van Slyke, 10 minutes).

10.00 " required 4.50 cc. 0.1 N HCl (Kjeldahl).

This gives an amino nitrogen:total nitrogen ratio of 0.05. Hence the substance was hydrolyzed to the extent of 10 per cent.

STUDIES ON RACEMIZATION

X. ACTION OF ALKALI ON KETOPIPERAZINES AND PEPTIDES

By P. A. LEVENE, ROBERT E. STEIGER, AND R. E. MARKER
(From the Laboratories of The Rockefeller Institute for Medical Research,
New York)

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The various ketopiperazines studied in two previous works¹ on racemization by alkali were unsymmetric. These were composed of an optically active amino acid combined in each case with glycine. It was pointed out that the extent of racemization found, representing the possible limit reached in each case because of hydrolysis, increased with the stability of the ketopiperazine and it was observed that the racemization was more or less complete when the reactions were carried out in alkaline pyridine-water media. In the case of unsymmetric ketopiperazines, undergoing hydrolysis² simultaneously with racemization, matters are naturally complicated by the possibility of a ring opening in two different ways, hence by the fact that two secondary reactions of this nature, proceeding at different rates, take place.³ This point has to be kept in mind in any comparison of the effects produced by the alkali on a series of ketopiperazines of this type. Our observations have now been extended to symmetrically constituted anhydrides composed of two optically active amino acids.

From Table I it is seen that dextro-alanyl-dextro-alanine anhydride is racemized to the extent of 80 per cent under the conditions of the experiment. This limit is reached long before the expiration of the time interval of 48 hours adopted in all our work on ketopiperazines. A corresponding fraction of fully or half active

¹ Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, **76**, 299 (1928); **86**, 703 (1930).

² Levene, P. A., Rothen, A., Steiger, R. E., and Osaki, M., *J. Biol. Chem.*, **86**, 723 (1930).

³ See Fischer, E., and Schrauth, W., *Ann. Chem.*, **354**, 21 (1907).

anhydride undergoes hydrolysis to the dipeptides. These, as is shown by the experiment performed on dextro-alanyl-dextro-alanine (see Table I), retain their entire optical activity even on greatly prolonged contact with alkali. Of particular interest is the case of dextro-leucyl-dextro-leucine anhydride. This compound was found to be practically insoluble in water and but sparingly soluble in a pyridine (57 per cent by volume)-water mixture. A solution, 0.04 M in respect to anhydride and 0.08 M in respect to alkali, was prepared and allowed to stand at 25°. Under

TABLE I

Action of 0.2 N Sodium Hydroxide (2 Mols per Mol of Substance) at 25° on Dextro-Alanine Anhydride and on the Corresponding Dipeptide

Compound	Time of action of alkali	[α] _D ²⁵ of alanine in hydrolysate with 20 per cent HCl (+NaCl)		Racemization
		Treated	Blank	
	days	degrees	degrees	per cent
Dextro-alanyl-dextro-alanine anhydride	2	+2.77	+13.54	80
Dextro-alanyl-dextro-alanine	2	+13.50*	+13.7 ₉	0
	8	+13.7 ₆		

* Value somewhat low. Hydrolysis not quite complete. The dipeptide is strongly levorotatory in (10 per cent) hydrochloric acid solution; it is only slowly hydrolyzed at 100°. See Fischer, E., *Ber. chem. Ges.*, **39**, 466 (1906).

these conditions no hydrolysis of the anhydride takes place⁴ and the progress of racemization can be followed by the decrease in rotation of the solution. From Fig. 1 it is seen that 80 per cent of the anhydride is racemized in the course of about 2 hours and that the racemization is complete after 6 hours (see also Experiment 4, recorded in Table III). A control experiment was made with a similar solution containing no sodium hydroxide. No change in rotation was observed at 25° in the course of several hours. These experiments show conclusively that the racemization values previously observed¹ for other anhydrides in alkaline

⁴ See also Fischer, E., *Ber. chem. Ges.*, **38**, 609 (1905) and Fischer, E., and Schrauth, W., *Ann. Chem.*, **354**, 27 (1907).

pyridine-water solutions were high due to the fact (established experimentally on glycyl-levo-leucine anhydride²) that the rates of hydrolysis of these ketopiperazines were very low in such media. In the case of glycyl-levo-phenylglycine anhydride the rate of racemization was undoubtedly very high. Inasmuch as complete racemization had been observed with this compound it must be inferred that like dextro-leucine anhydride it did not suffer any

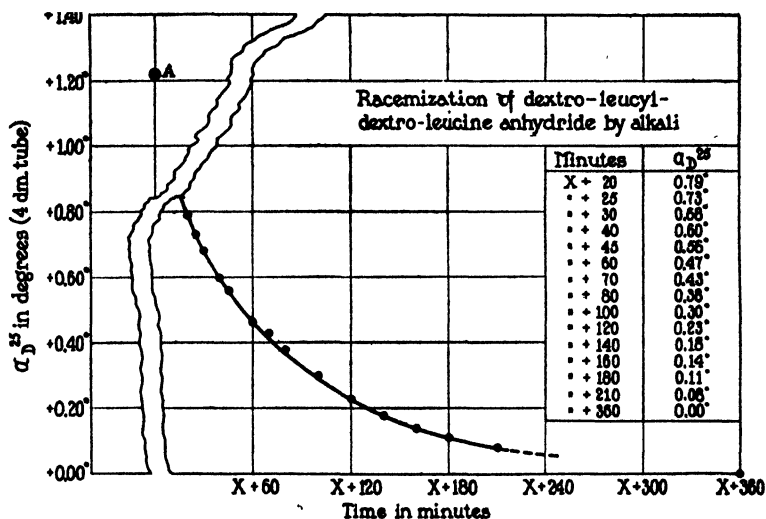


FIG. 1. Showing the decrease in rotation observed with 0.002 mol of dextro-leucyl-dextro-leucine anhydride and 0.004 mol of sodium hydroxide in a pyridine (57 per cent by volume) -water mixture. Volume of the solution, 50.0 cc. at 25°. Concentration of sodium hydroxide, 0.08 N. Concentration anhydride, 0.04 M. A is the rotation of the control solution containing the 90 per cent active material used here but no sodium hydroxide (+1.22°). X is the very short time interval in which the activity of an optically pure anhydride would drop to that of our starting material.

hydrolysis in the course of the time necessary for the primary reaction to proceed to completion. With these experiments our work on the racemization of ketopiperazines has been brought to the following conclusion. *Ketopiperazines undergo rapid racemization in alkaline solutions. The reaction is complete whenever there is no hydrolysis of the ketopiperazine.*

Polypeptides behave differently. In previous work on the

action of alkali on peptides, di-, tri-, and tetrapeptides composed of glycine and levo-alanine were studied.⁵ They did not possess the high resistance towards hydrolysis exhibited by the leucine derivatives now examined. The phenomena observed with this latter type of peptides are more readily interpreted.

From Table II it is seen that the dipeptide dextro-leucyl-dextro-leucine is not racemized by 10 equivalents of 1.0 N alkali at 25° and that similarly the two tripeptides listed there retain their full optical activity on prolonged contact with 2 equivalents of 0.2 N sodium hydroxide. In more concentrated alkali (10 equiva-

TABLE II
Racemization of Peptides in Alkaline Solutions at 25°

Compound	Alkali used	$[\alpha]_D^{25}$ of amino acids in hydrolysate with 20 per cent HCl (+NaCl)			Racemization after	
	NaOH	2 days	8 days	Blank	2 days	8 days
	N	degrees	degrees	degrees	per cent	per cent
Dextro-leucyl-dextro-leucine	1.0*	-15.2	-15.1	-15.2	0	0
Dextro-leucyl-dextro-leucyl-dextro-leucine	0.2†		-15.5	-15.5		0
	1.0*		-14.9			4
Levo-alanyl-dextro-leucyl-dextro-leucine	0.2†		-14.0	-13.9		0
	1.0*		-13.2			5

* 10 mols of NaOH per mol of substance.

† 2 mols of NaOH per mol of substance.

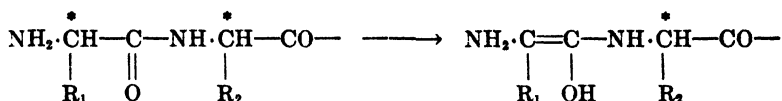
lents of 1.0 N sodium hydroxide) there is under otherwise identical conditions a slight racemization of these tripeptides. Thus the degree of racemization increases (however slightly) with the concentration of the alkali used and with the length of the peptidic chain.

According to Dakin,⁶ the racemization of proteins by alkali at low temperatures is due to a keto-enol tautomerism of the

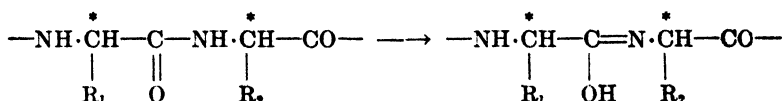
⁵ Levene, P. A., and Pfaltz, M. H., *J. Gen. Physiol.*, **8**, 183 (1925); *J. Biol. Chem.*, **68**, 277 (1926); **70**, 219 (1926).

⁶ See Dudley, H. W., and Woodman, H. E., *Biochem. J.*, **12**, 341 (1918).

$>\overset{*}{\text{CH}}-\text{CO}-$ groups in the protein complex. Wherever this change occurs the asymmetry of that particular grouping will be destroyed. Dakin⁷ also expressed the opinion that it appeared likely that the conditions necessary for racemization of an amino acid group required the attachment of other groups to both the amino and the carboxyl radical of this unit. Hence racemization would be observed only with systems composed of at least three amino acids. Our findings substantiate the predictions of Dakin. It may be said, however, that there is no theoretical reason apparent militating against the possibility of a complete racemization in any di- or polypeptide of that terminal amino acid carrying the free amino group.



The other type of enolization, not involving the hydrogens attached to the asymmetric carbon atoms, believed to occur in proteins, is the following.



The view has been expressed in a previous work⁸ that enolization of the second type probably takes place much more readily in polypeptides than in ketopiperazines. An inspection of the schemes above shows that when ready enolization (with formation of more or less stable sodium salts) of the latter type occurs, there is proportionally less racemization to be expected since both these phenomena involve the same central carbonyl groups and hence are antagonistic. A certain minimum concentration of alkali seems to be required to bring about these changes.

⁷ Dakin, H. D., *J. Biol. Chem.*, **13**, 362 (1912-13).

⁸ Levene, P. A., Bass, L. W., Steiger, R. E., and Bencowitz, I., *J. Biol. Chem.*, **72**, 815 (1927).

EXPERIMENTAL

1: Preparation of Compounds

Dextro-Alanyl-Dextro-Alanine

This peptide⁹ is most conveniently¹⁰ obtained in the following way.¹¹ Crude *d,l*- α -bromopropionyl-dextro-alanine (from *d,l*- α -bromopropionyl chloride and dextro-alanine) was converted by amination into a mixture of dextro-alanyl-dextro-alanine and levo-alanyl-dextro-alanine. The separation of the two peptides is easily accomplished inasmuch as but the dextro-alanyl-dextro-alanine crystallizes when a large excess of alcohol is added to an aqueous solution of the mixture. The crude peptide was then twice recrystallized in the usual way from water and alcohol.

No. 267.

4.850 mg. substance: 8.030 mg. CO₂ and 3.325 mg. H₂O.

4.320 " " : 0.680 cc. (29°, 758.5 mm.).

C₈H₁₂O₃N₂ (160.12). Calculated. C 44.97, H 7.55, N 17.50

Found. " 45.15, " 7.67, " 17.75

Moisture, 0.39 per cent

It had the following rotation.

$$[\alpha]_D^{25} = \frac{-5.43^\circ \times 100}{4 \times 6.4047} = -21.2^\circ \text{ (in water)}$$

0.9607 gm., dry, in 15.00 cc. of solution (0.4 M).

Fischer⁹ found $[\alpha]_D^{20} = -21.6^\circ$, employing a solution of 0.3523 gm. in water, the weight of the solution being 7.1448 gm. Abderhalden and Delgado y Mier¹⁰ also found $[\alpha]_D^{20} = -21.6^\circ$.

Dextro-Leucyl-Dextro-Leucine

A. *Levo- α -Bromoisocaproonyl-Dextro-Leucine*¹²—A solution of 26.3 gm. (0.2 mol) of dextro-leucine in 100 cc. of 2.0 N sodium hydroxide (0.2 mol) was cooled in an ice-water mixture during treatment

⁹ Fischer, E., *Ber. chem. Ges.*, **39**, 465 (1906).

¹⁰ Compare Abderhalden, E., and Delgado y Mier, J. J., *Fermentforschung*, **10**, 252 (1928-29).

¹¹ Fischer, E., and Schulze, A., *Ber. chem. Ges.*, **40**, 945, 952, 954 (1907).

¹² Compare with the indications of Fischer, E., and Koelker, A. H., *Ann. Chem.*, **354**, 39 (1907). See also Fischer, E., *Ber. chem. Ges.*, **39**, 2918 (1906).

with 43 gm. (0.2 mol) of levo- α -bromoisocapronyl chloride (from recrystallized levo-leucine) and 100 cc. of 2.0 N sodium hydroxide (0.2 mol), these reagents being added in equivalent proportions with continuous shaking. 10 cc. of ether and then 52 cc. of 5.0 N hydrochloric acid (0.26 mol) were added to the solution. The bromo compound precipitated as a pasty mass. The mixture was then shaken at room temperature until crystallization took place. The cake was then crushed in a mortar. The crystals were filtered off, washed with ice water, then with petroleic ether (40–60°); they were then washed with water and this was finally displaced by petroleic ether. The bromo compound was dried in a vacuum desiccator. Three such operations were made; the weights of the crude, dried materials were as follows: 55.8 gm. (first operation), 57.2 gm. (second operation), and 55.7 gm. (third operation). The average yield was 91 per cent of the theory. Fischer and Koelker's yield was only 80 per cent.

*B. Dextro-Leucyl-Dextro-Leucine*¹²—A mixture of 168.5 gm. of crude levo- α -bromoisocapronyl-dextro-leucine with 8 times this weight of ammonium hydroxide of sp. gr. of 0.90 was kept at room temperature for 6 days. The solution was diluted with water, clarified with norit, and concentrated under reduced pressure. It yielded thus several fractions of dipeptide. A large volume of alcohol was added to the filtrate of the last fraction and the mixture was evaporated to dryness. The residue was extracted with cold absolute alcohol. The dipeptide which had remained insoluble was filtered off, washed with absolute alcohol and with benzene. The various fractions of dipeptide were combined. Yield of air-dried material containing water of crystallization, 100 gm. This crude material was dissolved in 18.5 parts (by weight) of 85 per cent by weight alcohol at boiling temperature. Norit was added and the solution filtered while hot. The filtrate was cooled for 60 hours in ice. The crystals were filtered off, washed with absolute alcohol, and dried in the air. Yield, 51 gm. They were again recrystallized from boiling 85 per cent by weight alcohol in which, curiously enough, they were much less soluble than the original material. The solution saturated at boiling temperature was rapidly filtered with suction. Immediately crystallization took place; the mixture was then cooled in ice. The crystals were washed with absolute alcohol, dried in the air, and then kept for

several months in a vacuum desiccator over phosphorus pentoxide. They weighed 25.3 gm. (No. 315). This substance contained 7.74 per cent of moisture. This was established in the following way. When heated for 2½ hours to 100° under diminished pressure, the moist dipeptide lost 7.19 per cent of water. The drying was then interrupted inasmuch as according to Fischer the peptide is after this time partly converted into the corresponding anhydride. Two analyses of the material thus obtained were performed.

3.100 mg. substance:	6.655 mg. CO ₂ and	2.745 mg. H ₂ O.
3.558 " " "	7.660 " " "	3.190 " " "
C ₁₂ H ₂₄ O ₅ N ₂ (244.21). Calculated. C 58.97, H 9.91		
Found. " 58.54, " 9.90		
" 58.70, " 10.00		

It contained, therefore (C 58.62 per cent, mean), 99.41 per cent of dry dipeptide. Hence, the original substance (No. 315), contained 92.26 per cent of dry dipeptide or 7.74 per cent of water. The rotation was as follows:

$$[\alpha]_D^{25} = \frac{+2.51^\circ \times 100}{4 \times 4.5273} = +13.86^\circ \text{ (in 1.0 N NaOH)}$$

0.7371 gm. of moist dipeptide (0.6800 gm., dry) in 15.02 cc. of solution.

This material (dry) contained 98 per cent of dextro-leucine in combined form (see hydrolysis Experiment 15, recorded in Table IV). Fischer and Koelker¹³ found $[\alpha]_D^{20} = +13.16^\circ (\pm 0.2^\circ)$, employing a solution of 0.3810 gm. of moist dipeptide (0.3193 gm., dry) in 1.0 N sodium hydroxide, the weight of the solution being 3.5868 gm. Fischer¹⁴ found for levo-leucyl-levo-leucine $[\alpha]_D^{20} = -13.43^\circ (\pm 0.4^\circ)$, employing a solution of 0.1628 gm. of moist dipeptide (0.1512 gm., dry) in 1.0 N sodium hydroxide, the weight of the solution being 3.4877 gm. Abderhalden and Fleischmann¹⁵ found for levo-leucyl-levo-leucine $[\alpha]_D^{20} = -13.95^\circ$ in 1.0 N sodium hydroxide.

Another sample of dipeptide (No. 263) of another preparation,

¹³ Fischer, E., and Koelker, A. H., *Ann. Chem.*, **354**, 48 (1907).

¹⁴ Fischer, E., *Ber. chem. Ges.*, **39**, 2919 (1906).

¹⁵ Abderhalden, E., and Fleischmann, R., *Fermentforschung*, **9**, 526 (1926-28).

after having been kept for a long period in a vacuum desiccator over phosphorus pentoxide, was then found to contain exactly 1 mol of water of crystallization. It lost 5.72 per cent of water on heating for $2\frac{1}{2}$ hours to 100° .

4.445 mg. substance: 9.495 mg. CO_2 and 3.850 mg. H_2O .
 $\text{C}_{12}\text{H}_{24}\text{O}_5\text{N}_2$ (244.21). Calculated. C 58.97, H 9.91
 Found. " 58.25, " 9.69

The dried material thus contained 98.78 per cent of dipeptide and hence the original substance (No. 263) 93.13 per cent of dry dipeptide or 6.87 per cent of water.

$\text{C}_{12}\text{H}_{24}\text{O}_5\text{N}_2 + 1 \text{ H}_2\text{O}$ (262.23). Calculated. H_2O 6.87 per cent
 Found. " 6.87 " "

Dextro-Alanyl-Dextro-Alanine Anhydride

It was prepared by heating the methyl ester of dextro-alanine for 12 hours at 100° .¹⁶ The crude product was thoroughly washed with ether. It was then purified by recrystallization from 10 times its weight of boiling water.

No. 313.

4.380 mg. substance: 8.135 mg. CO_2 and 2.795 mg. H_2O .
 3.060 " " : 0.537 cc. N (25° , 756.2 mm.).
 $\text{C}_6\text{H}_{10}\text{O}_2\text{N}_2$ (142.10). Calculated. C 50.67, H 7.09, N 19.72
 Found. " 50.56, " 7.14, " 19.98
 Moisture, 0.45 per cent

It had the following rotation.

$$[\alpha]_D^{25} = \frac{-1.57^{\circ} \times 100}{4 \times 1.4214} = -27.6^{\circ} \text{ (in water)}$$

0.2132 gm., dry, in 15.00 cc. of solution (0.1 M).

Fischer¹⁶ found for dextro-alanyl-dextro-alanine anhydride prepared from the ester of dextro-alanyl-dextro-alanine, $[\alpha]_D^{20} = -28.8^{\circ}$ ($\pm 0.5^{\circ}$), employing a solution of 0.2741 gm. in water, the weight of the solution being 12.8787 gm., and the constant value of $[\alpha]_D^{20} = -27^{\circ}$ for the anhydride obtained by heating the methyl ester of dextro-alanine for 12 hours at 100° .

¹⁶ Fischer, E., *Ber. chem. Ges.*, **39**, 468 (1906).

Dextro-Leucyl-Dextro-Leucine Anhydride

It was prepared from dextro-leucyl-dextro-leucine (an optically impure material was used) by the procedure established by Fischer¹⁷ for levo-leucyl-levo-leucine anhydride. It was purified by recrystallization from boiling alcohol. Two large fractions were obtained.¹⁸ The analysis of the first fraction was as follows:

No. 316.

4.860 mg. substance: 11.372 mg. CO₂ and 4.125 mg. H₂O.

4.960 " " : 0.546 cc. N (28°, 757.3 mm.).

C₁₂H₂₂O₂N₂ (226.20). Calculated. C 63.66, H 9.80, N 12.39

Found. " 63.81, " 9.90, " 12.43

Moisture, 0.37 per cent

This material had the following rotation.

$$[\alpha]_D^{25} = \frac{+4.02^\circ \times 100}{4 \times 2.2660} = +44.3^\circ \text{ (in glacial acetic acid)}$$

0.3399 gm., dry, in 15.00 cc. of solution.

It contained from 95 to 96 per cent of pure dextro-leucine anhydride, inasmuch as (see for a check hydrolysis Experiment 14, recorded in Table IV) Fischer and Koelker¹⁸ $[\alpha]_D^{20} = +48.67^\circ$ ($\pm 0.3^\circ$), employing a solution of 0.2476 gm. in glacial acetic acid, the weight of the solution being 2.8424 gm. Other preparations by these authors had lower rotations.

Dextro-Leucyl-Dextro-Leucyl-Dextro-Leucine

A. *Levo- α -Bromoisocaproonyl-Dextro-Leucyl-Dextro-Leucine*—A solution of 29.4 gm. (dry, 0.12 mol) of dextro-leucyl-dextro-leucine (34.8 gm. of dipeptide containing 15 per cent of moisture) in 120 cc. of 1.0 N sodium hydroxide (0.12 mol) was cooled in an ice-water mixture during treatment with 33.4 gm. (0.156 mol) of levo- α -bromoisocaproonyl chloride (from recrystallized levo-leucine) and 180 cc. of 1.0 N sodium hydroxide (0.18 mol), these reagents being added in equivalent proportions with continuous shaking. A crystalline precipitate of the bromo compound was obtained at once on addition of 46 cc. of 5.0 N hydrochloric acid (0.23 mol). It was

¹⁷ Fischer, E., *Ber. chem. Ges.*, **39**, 2920 (1906).

¹⁸ Fischer, E., and Koelker, A. H., *Ann. Chem.*, **354**, 49 (1907).

washed with cold water and then with petroleic ether. It weighed 46 gm. (yield, 91 per cent of the theory). This crude material was recrystallized three times in the following way. Water was added to the hot alcoholic solution to incipient turbidity. The bromo compound crystallized on cooling. It was converted as described below into the tripeptide fraction No. 268. The bromo compound contained in the mother liquors of crystallization of the preceding was recovered; it was also aminated, but separately. It yielded the tripeptide fraction No. 269.

B. Dextro-Leucyl-Dextro-Leucyl-Dextro-Leucine. Main Fraction—A mixture of 21.5 gm. of the recrystallized bromo compound with 10 times this weight of ammonium hydroxide of sp. gr. 0.90 was kept at 37° for 3 days, during which time it was occasionally shaken. The solution was diluted with an equal volume of water; it was clarified with norit, and evaporated to dryness under reduced pressure, octyl alcohol being added from time to time to prevent frothing. The residue was suspended in lukewarm absolute alcohol; it was filtered off after cooling, and thoroughly washed with absolute alcohol; it was then washed successively with ether, water, alcohol, and finally with ether. This crude tripeptide weighed 15 gm. It was suspended in 450 gm. of absolute alcohol; 15 gm. of ammonium hydroxide of sp. gr. 0.90 and then norit were added. The filtered solution was concentrated under reduced pressure. As the ammonia was removed, the tripeptide precipitated. The suspension was left to cool in the ice box. The crystals were washed with alcohol, then with ether and air-dried; they weighed 15 gm. This material was once more purified in the same way. A sample of the substance thus obtained (No. 268) was dried for exactly 2½ hours at 100° under diminished pressure (loss of weight, 4.10 per cent) and two analyses of this partly dried material (*cf.* case of dextro-leucyl-dextro-leucine) were performed.

3.577 mg. substance: 7.615 mg. CO₂ and 3.220 mg. H₂O.

4.232 " " : 9.037 " " 3.845 " "

C₁₈H₃₁O₄N₃ (357.31). Calculated. C 60.45, H 9.87

Found. " 58.06, " 10.07

" 58.23, " 10.16

The dried material contained, therefore (C 58.15 per cent, mean), 96.20 per cent of dry tripeptide. Hence the original substance

(No. 268) contained 92.26 per cent of dry tripeptide or 7.74 per cent of moisture. The rotation of the tripeptide was as follows:

$$[\alpha]_D^{25} = \frac{+11.87^\circ \times 100}{4 + 6.5440} = +45.4^\circ \text{ (in 1.010 N NaOH)}$$

0.9816 gm., dry, in 15.00 cc. of solution.

Abderhalden and Fleischmann¹⁹ found for levo-leucyl-levo-leucyl-levo-leucine $[\alpha]_D^{20} = -51.36^\circ$, employing a solution of 0.3115 gm. in 1.0 N sodium hydroxide, the volume of the solution being 10 cc. Thus at first sight, if it is assumed that the rotation of the optically pure tripeptide (composed of dextro acids) is $[\alpha]_D^{20} = +51.36^\circ$, it would seem that our material was only 88 per cent active. That such a comparison is not permissible is clearly shown by the result of the hydrolysis experiment (Experiment 16, recorded in Table IV). The rotation of the leucine contained in the acid hydrolysate of this tripeptide was $[\alpha]_D^{25} = -15.5^\circ$. Since Fischer and Warburg²⁰ found for pure levo-leucine $[\alpha]_D^{20} = +15.8^\circ$, in 20 per cent hydrochloric acid, the tripeptide contained 99 per cent of dextro-leucine.

Second Fraction (No. 269)—It was prepared from a seemingly less pure levo- α -bromoisocapronyl-dextro-leucyl-dextro-leucine (see that paragraph). It was purified in the same way as the main fraction above. It had the following rotation.

$$[\alpha]_D^{25} = \frac{+11.97^\circ \times 100}{4 \times 6.5087} = +46.0^\circ \text{ (in 1.010 N NaOH)}$$

0.9763 gm., dry, in 15.00 cc. of solution (concentration determined by Kjeldahl. 5.00 cc. of this solution contained 0.7655 gm. of nitrogen).

Levo- α -Bromopropionyl-Dextro-Leucyl-Dextro-Leucine

A solution of 29.4 gm. (dry, 0.12 mol) of dextro-leucyl-dextro-leucine (34.8 gm. of dipeptide containing 15 per cent of moisture) in 120 cc. of 1.0 N sodium hydroxide (0.12 mol) was cooled in an ice-water mixture during treatment with 27 gm. (0.156 mol) of levo- α -bromopropionyl chloride (from recrystallized dextro-alanine) and

¹⁹ Abderhalden, E., and Fleischmann, R., *Fermentforschung*, **9**, 528 (1928-28).

²⁰ Fischer, E., and Warburg, O., *Ber. chem. Ges.*, **38**, 4004 (1905).

180 cc. of 1.0 N sodium hydroxide (0.18 mol), these reagents being added in equivalent proportions with continuous shaking. 46 cc. of 5.0 N hydrochloric acid were then added. The precipitate of crude bromo compound weighed after drying 40 gm. (yield, 88 per cent of the theory). It was purified in the following way. 40 gm. were dissolved in 210 gm. of hot 35 per cent by weight alcohol. The crystals obtained on cooling in ice water were washed with 35 per cent alcohol. They weighed 38.5 gm. These were dissolved in 158 gm. of 45 per cent by weight alcohol and gave 36 gm. of a purer material. This was dissolved in 216 gm. (6 parts) of 55 per cent by weight alcohol. It gave 28 gm. which finally were dissolved in 168 gm. (6 parts) of 50 per cent by weight alcohol. 25 gm. of bromo compound were obtained. It was dried in a vacuum desiccator over phosphorus pentoxide and soda-lime. It melted and decomposed at 180° (uncorrected).

No. 270.

4.065 mg. substance: 7.135 mg. CO₂ and 2.690 mg. H₂O.

0.1000 gm. " : 5.05 cc. 0.1 N HCl (Kjeldahl).

0.1120 " " : 0.0552 gm. AgBr (Carius).

C₁₈H₂₇O₄N₂Br (379.16). Calculated. C 47.47, H 7.18, N 7.39, Br 21.08

Found. " 47.86, " 7.40, " 7.07, " 20.97

Moisture, none

It had the following rotation.

$$[\alpha]_D^{25} = \frac{+14.06^\circ \times 100}{4 \times 7.5833} = +46.35^\circ \text{ (in absolute alcohol)}$$

1.1375 gm. in 15.00 cc. of solution (0.2 M).

Levo-Alanyl-Dextro-Leucyl-Dextro-Leucine

A mixture of 21.5 gm. of purified bromo compound, $[\alpha]_D^{25} = +46.35^\circ$, with 10 times this weight of ammonium hydroxide of sp. gr. 0.90 was kept at 37° for 3 days, during which time it was occasionally shaken. The solution was diluted with an equal volume of water. It was clarified with norit and evaporated to dryness under reduced pressure, octyl alcohol being added from time to time to prevent frothing. The residue was dried by reevaporation with absolute alcohol; the suspended material was filtered off and was washed with absolute alcohol; it was then washed successively

with water, alcohol, and finally with ether. This crude tripeptide weighed 14 gm. It was suspended in 420 gm. of absolute alcohol, 14 gm. of ammonium hydroxide of sp. gr. 0.90 were added, and the mixture was shaken at room temperature until practically all had dissolved. Norit was then added and the filtered solution was concentrated under reduced pressure. As the ammonia was removed, the tripeptide precipitated as a gel. It became crystalline when left to stand overnight in the ice box. The crystals were filtered off, washed with absolute alcohol and with anhydrous ether, and dried in the air; yield, 11 gm. This material was once more purified in the same way; yield, 10 gm.

No. 271.

3.540 mg. substance: 7.435 mg. CO₂ and 2.895 mg. H₂O.

6.275 " " : 0.742 cc. N (23°, 752 mm.).

C₁₅H₂₃O₄N₃ (315.26). Calculated. C 57.10, H 9.27, N 13.33

Found. " 57.27, " 9.15, " 13.49

Moisture, 1.32 per cent (100°)

It had the following rotation.

$$[\alpha]_D^{25} = \frac{+7.82^\circ \times 100}{4 \times 3.1527} = +62.0^\circ \text{ (in 1.010 N NaOH)}$$

0.4729 gm., dry, in 15.00 cc. of solution (0.1 M).

Another fraction of tripeptide was prepared by amination of the bromo compound contained in the combined mother liquors of crystallization of the purified product. It was purified in the same way as the main fraction. The analysis was as follows:

No. 272.

4.760 mg. substance: 9.980 mg. CO₂ and 3.860 mg. H₂O.

4.425 " " : 0.520 cc. N (24° 754.4 mm.).

C₁₅H₂₃O₄N₃ (315.26). Calculated. C 57.10, H 9.27, N 13.33

Found. " 57.17, " 9.07, " 13.40

Moisture, 0.30 per cent

It had the following rotation.

$$[\alpha]_D^{25} = \frac{+7.84^\circ \times 100}{4 \times 3.1527} = +62.2^\circ \text{ (in 1.010 N NaOH)}$$

0.4729 gm., dry, in 15.00 cc. of solution (0.1 M).

TABLE III
Action of Sodium Hydroxide at 25° on Ketopiperazines, Dipeptides, and Tripeptides

Compound	Dextro-alanyl-dextro-alanine anhydride		Dextro-alanyl-dextro-alanine	Dextro-leucyl-dextro-leucine anhydride	Dextro-leucyl-dextro-leucine	Dextro-leucyl-dextro-leucyl-dextro-leucine	Levo-alanyl-dextro-leucyl-dextro-leucine
	Experiment No.	1	2 and 3	4	5 and 6	7 and 8	9 and 10
Amount of substance (0.0025 mol, dry), gm.		0.3553	0.4003 (2) 0.4003 (3)	0.4524 (0.002 mol)	0.6106 (5) 0.6106 (6)	0.8933 (7) 0.8933 (8)	0.7882 (9) 0.7882 (10)
Reagent used		∞ 19 cc. water + 5.00 cc. 1.0 N NaOH (0.005 mol) + water. Volume, 25.0 cc. (25°)		28.50 cc. pyridine + 16 cc. water + 4.00 cc. 1.0 N NaOH (0.004 mol) + water. Volume, 50.0 cc.	25.0 cc. 1.0 N NaOH (0.025 mol)	∞ 19 cc. water + 5.00 cc. 1.0 N NaOH (0.005 mol) + water. Volume, 25.0 cc. (25°)	
Action of alkali at 25°, days	2	2 (2) 8 (3)	0.3407 ₆ 0.3371 (3)	2 0.2744	2 (5) 8 (6)	8 (7) 8* (8)	8 (9) 8 (10)
Total N in hydrolysate with 20 cc. of 20 per cent HCl, gm. per 100 cc.	0.3407 ₆	0.3407 ₆ (2) 0.3371 (3)	0.3407 ₆ (2) 0.3371 (3)	0.2744	0.3374 (5) 0.3318 (6)	0.5191 ₂ (7) 0.4916 ₆ (8)	0.5076 ₄ (9) 0.4916 ₆ (10)
α_1^{D} (4 dm. tube), degrees	+0.24	+1.17 (2) +1.18 (3)	+1.17 (2) +1.18 (3)	0.00	-1.92 (5) -1.87 ₆ (6)	-3.01 (7) -2.75 (8)	-2.38 (9) -2.17 (10)
$[\alpha]_D^{25}$ of amino acids in solution, degrees	+2.77	+13.5 ₆ (2) +13.7 ₆ (3)	+13.5 ₆ (2) +13.7 ₆ (3)	0.0 ₆	-15.2 ₆ (5) -15.1 ₆ (6)	-15.4 ₉ (7) -14.9 ₄ (8)	-14.0 ₂ (9) -13.2 ₆ (10)

* The solution deposited long needles of an unidentified substance. Compare Abderhalden, E., and Fleischmann, R., *Fermentforschung*, 9, 532 (1928-28).

TABLE IV
Hydrolysis of Ketopiperazines, Dipeptides, and Tripeptides by Hydrochloric Acid at 100°

Compound	Dextro-alanyl-dextro-alanine anhydride	Dextro-alanyl-dextro-alanine	Dextro-leucyl-dextro-leucine anhydride	Dextro-leucyl-dextro-leucine	Dextro-leucyl-dextro-leucyl-dextro-leucine	L- α -alanyl-dextro-leucyl-dextro-leucine
Experiment No.	11 and 12	13	14	15	16	17 and 18
Amount of substance (0.0025 mol, dry), gm.	0.3553 (11) 0.3553 (12)	0.4003	0.4524 (0.002 mol)	0.6106	0.8933	0.7882 (17) 0.7882 (18)
Amount of NaCl added, gm.	0.2923 0.005	0.2923 0.005	0.2339 0.004	0.2923 0.005	0.2923 0.005	0.2923 0.005
" " " mol						
Total N in hydrolysate with 20 cc. 20 per cent HCl, gm. per 100 cc.	0.3455 ₂ (11) 0.3449 ₆ (12)	0.3435 ₆	0.2758	0.3435 ₆	0.5283 ₆	0.5073 ₈ (17) 0.5157 ₆ (18)
α_D^{25} (4 dm. tube), degrees	+1.19 (11) +1.19 (12)	+1.20 ₆	-1.48	-1.95 ₅	-3.07	-2.36 (17) -2.38 (18)
$[\alpha_D]^{25}$ of amino acids in solution, degrees	+13.5 ₄ (11) +13.5 ₆ (12)	+13.7 ₉	-14.3 ₃	-15.2 ₀	-15.5 ₂	-13.9 ₁ (17) -13.8 ₀ (18)

2. Action of Sodium Hydroxide on Ketopiperazines, Dipeptides, and Tripeptides (Racemization)

Alkaline solutions of the compounds prepared were allowed to stand for certain lengths of time in a thermostat kept at 25°. All the specific data are recorded in Table III. The solutions were then acidified and concentrated to dryness under reduced pressure in the way indicated in a previous work.¹ The dry residues were taken up in 20 cc. of 20 per cent hydrochloric acid. The test-tubes were sealed and immersed for 24 hours in a steam-heated water bath at 100°. The solutions or suspensions obtained were filtered in order to remove insoluble particles or undissolved sodium chloride. The total nitrogen was determined on 5 cc. and the amino nitrogen on 1 cc. of solution. For all other details of technique, see the preceding paper¹ of this series.

The extent of racemization by alkali is given in per cent by the ratio

$$\frac{[\alpha_0]_D^{25} - [\alpha_1]_D^{25}}{[\alpha_0]_D^{25}} \times 100$$

The values $[\alpha_0]_D^{25}$ of the blanks are given in Table IV.

3. Action of Hydrochloric Acid on Ketopiperazines, Dipeptides, and Tripeptides (Hydrolysis)

The various compounds prepared were hydrolyzed with 20 per cent hydrochloric acid in the presence of sodium chloride. The sealed tubes were immersed for 24 hours in a steam-heated water bath at 100°. All the experimental data are recorded in Table IV. The values $[\alpha_0]_D^{25}$ were thus obtained.

THE RING STRUCTURE OF NORMAL METHYLRIBOSIDE

By P. A. LEVENE AND R. STUART TIPSON

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York)

(Received for publication, June 29, 1931)

It has been shown that oxidation of the normal trimethyl derivatives of xylose,¹ arabinose,² and lyxose³ (or simultaneous hydrolysis and oxidation of their methyl glycosides), by concentrated nitric acid, leads in each case to the formation of a trimethoxyglutaric acid. This implies that in each of these three trimethyl pentoses the methoxyl groups are attached to the second, third, and fourth carbon atoms and that the oxygen of the ring must be attached to the fifth carbon atom; *i.e.*, they are all pyranose derivatives.

Similar experimental methods have now been applied to determine the structure of normal methylriboside and definite proof has been obtained to the effect that this substance and its derivatives are of the pyranose type and thus conform to the general rule followed by normal sugar derivatives.

The normal trimethyl ribose required for the oxidation experiments was prepared by methylating a syrupy methylriboside which had been subjected to prolonged treatment with 1.5 per cent methyl alcoholic hydrogen chloride to insure complete transformation of any methylribofuranoside.

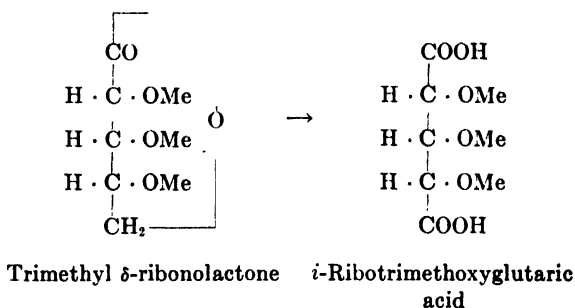
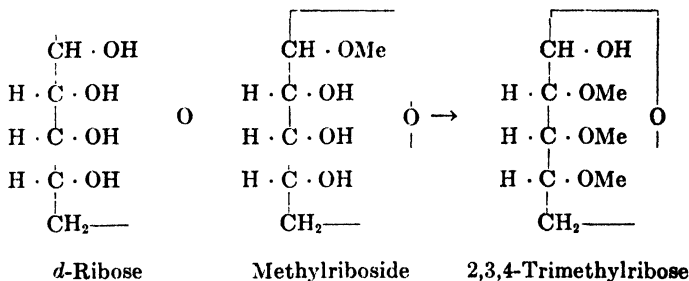
Hydrolysis of this trimethyl methylriboside gave crystalline trimethyl ribose; m.p., 85°. The constitution of this substance as 2, 3, 4-trimethyl ribose followed at once from a study of its oxidation by nitric acid; and also from the nature of the trimethyl ribonolactone obtained from it by the action of bromine water.

¹ Hirst, E. L., and Purves, C. B., *J. Chem. Soc.*, **123**, 1352 (1923).

² Hirst, E. L., and Robertson, G. J., *J. Chem. Soc.*, **127**, 358 (1925).
McOwan, G., *J. Chem. Soc.*, 1747 (1926).

³ Hirst, E. L., and Smith, J. A. B., *J. Chem. Soc.*, 3147 (1928).

The lactone displayed the behavior typical of a δ -lactone, since polarimetric observations during its hydrolysis in aqueous solution showed that equilibrium was attained in about 191 hours and that some 44 per cent of the lactone then remained unchanged. It is similar in its rate of hydrolysis to tetramethyl δ -mannonolactone and trimethyl δ -rhamnonolactone to which it is related in the configuration of carbon atoms (2) and (3). In addition, this lactone affords a further striking example of the profound influence⁴ exerted by the solvent upon the specific rotation of methylated sugar lactones having *cis* methoxyl groups attached to carbon atoms (2) and (3). When oxidized with concentrated nitric acid the sugar gave exclusively *i*-ribotrimethoxyglutaric acid, which was isolated in the form of its dimethyl ester.



EXPERIMENTAL

Preparation of Trimethyl Methylriboside—Methylriboside was prepared as previously described.⁵ 10.9 gm. of syrupy methyl-

⁴ Haworth, W. N., Hirst, E. L., and Smith, J. A. B., *J. Chem. Soc.*, 2659 (1930).

⁵ Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, 92, 109 (1931).

riboside were dissolved in 20 cc. of acetone and treated in the usual manner with 44 cc. of dimethyl sulfate and 86 cc. of 30 per cent aqueous sodium hydroxide at 55–70°, the temperature finally being raised to 100° for 30 minutes. Methylation of the product was completed by the use of Purdie's reagents and the resulting non-reducing syrup (13.1 gm.) was distilled giving two fractions.

(a) 9 gm. of a colorless, very mobile liquid boiling at 54° at 0.05 mm. (bath temperature, 73–77°), N_D^{21} , 1.4473.

It had the following composition.

4.810 mg. substance: 9.220 mg. CO_2 and 3.840 mg. H_2O .

6.896 " : 30.900 " AgI.

$\text{C}_7\text{H}_{14}\text{O}_5$. Calculated. C 52.4, H 8.8, OMe 60.2

Found. " 52.3, " 8.9, " 59.2

Its rotation was as follows:

$$[\alpha]_D^{24} = \frac{-1.75^\circ \times 100}{2 \times 2.501} - 35.0^\circ \text{ (in water)}$$

$$\frac{-1.12^\circ \times 100}{2 \times 2.316} - 24.2^\circ \text{ (in methyl alcohol)}$$

The substance, which possessed typical glucosidic properties, resisted all attempts to crystallize it.

(b) 3.5 gm. of a colorless mobile liquid boiling at 55–60° at 0.06 mm. (bath temperature, 76–98°), N_D^{23} , 1.4470.

4.220 mg. substance: 18.397 mg. AgI.

$\text{C}_7\text{H}_{14}\text{O}_5$. Calculated. OMe 60.2 Found. OMe 57.6

The second fraction was evidently not completely methylated, so was not used in the following work.

Preparation of 2, 3, 4-Trimethyl Ribose—4.0 gm. of trimethyl methylriboside were dissolved in 100 cc. of N/15 hydrochloric acid. The absence of furanose derivatives was shown by the negligible rate of hydrolysis at 90°. Sufficient concentrated hydrochloric acid was now cautiously added, with vigorous stirring, to raise the concentration to approximately 6 per cent hydrochloric acid and the resulting solution was heated at 98°. In the course of 1 hour the specific rotation altered from $[\alpha]_D^{24} = -35.7^\circ$ to a constant value $[\alpha]_D^{24} = -10.0^\circ$. The hydrochloric acid was then

partly neutralized with lead carbonate and the neutralization completed with barium carbonate. The mixture was filtered, the filtrate evaporated to dryness, and the trimethyl ribose was extracted by means of chloroform. Removal of solvent gave 3.5 gm. of syrupy product. This was dissolved in dry ether and on cautious addition of petroleum ether (30–40°) trimethyl ribose commenced crystallizing immediately in clusters of long fine needles, crystallization being complete in a few minutes; m.p., 80°. It was recrystallized from a mixture of ether and petroleum ether at 0°; m.p., 85–86°. Weight, 2.82 gm. It was strongly reducing towards boiling Fehling's solution and had a bitter taste. It had the following composition.

4.609 mg. substance: 8.440 mg. CO₂ and 3.457 mg. H₂O.

4.155 " " : 15.450 " AgI.

C₆H₁₂O₅. Calculated. C 49.97, H 8.4, OMe 48.45
Found. " 49.94, " 8.4, " 49.08

Its rotation in water was

$$[\alpha]_D^{25} = \frac{-1.11^\circ \times 100}{2 \times 1.074} = -51.7^\circ \text{ (initial)}$$

$$[\alpha]_D^{25} = \frac{-0.86^\circ \times 100}{2 \times 1.074} = -40.0^\circ \text{ (final)}$$

In another hydrolysis experiment under the same conditions 3.82 gm. of trimethyl methylriboside gave 2.97 gm. of crystalline trimethyl ribose (83.4 per cent yield). During the hydrolysis the specific rotation changed from -34.4° to -9.7° .

Since the yield was not as high as might be expected and since the final rotation of the hydrolysis mixture did not correspond to the equilibrium rotation of pure trimethyl ribose, it seemed possible that decomposition, similar to that encountered by Carruthers and Hirst⁶ during the hydrolysis of trimethyl methylxyloside, might have occurred owing to the use of too concentrated an acid solution and too elevated a temperature. It was therefore decided to use 4 per cent hydrochloric acid as hydrolyst,⁷ and to follow the course of the reaction at 85° both by observation of the

⁶ Carruthers, A., and Hirst, E. L., *J. Chem. Soc.*, **121**, 2299 (1922).

⁷ Phelps, F. P., and Purves, C. B., *J. Am. Chem. Soc.*, **51**, 2443 (1929).

specific rotation and by quantitative estimation of the free sugar present after various time intervals.

Approximately 5 gm. of trimethyl methylriboside were weighed accurately into a volumetric flask and made to 100 cc. with 4 per cent hydrochloric acid. The specific rotation of the solution was then observed after which it was heated under a reflux condenser in a bath at 85°. At appropriate times the solution was rapidly cooled in ice to room temperature and its specific rotation again observed. The amount of free sugar present in an aliquot part was then determined by the method of Willstätter and Schudel⁸ as modified by Goebel,⁹ the sample first being rendered neutral

TABLE I
Course of the Hydrolysis of Trimethyl Methylriboside

Time	$[\alpha]_D^{27}$	Trimethyl pentose formed
<i>hrs.</i>	<i>degrees</i>	<i>per cent</i>
0 0	-43.6	0.0
0.5	-41.1	
1.5	-33.6	
2.0	-31.0	86.5
2.5	-28.5	90.7
3.0	-26.6	93.3
3.5	-24.7	98.1

by titration with 0.1 N sodium hydroxide. That no appreciable evaporation occurred, during the period of heating under the reflux, was indicated by the constancy of this initial titer. The results are given in Table I.

From these results it seems probable that the presence of hydrochloric acid considerably modifies the specific rotation of trimethyl ribose in water.

Oxidation of Crystalline 2, 3, 4-Trimethyl Ribose—2.82 gm. of crystalline trimethyl ribose were dissolved in 20 cc. of water and 3 cc. of bromine were added, in portions of 0.5 cc., during 4 days at 38°. The excess bromine was then removed by aeration, silver oxide was added until all the mineral acid had been neutralized,

⁸ Willstätter, R., and Schudel, G., *Ber. chem. Ges.*, **51**, 780 (1918).

⁹ Goebel, W. F., *J. Biol. Chem.*, **72**, 812 (1927).

the mixture was filtered, and the silver salts well washed with hot water. To the combined aqueous solution was added dilute hydrochloric acid from a burette until all the silver in solution had been precipitated. The mixture was filtered and the filtrate evaporated to a thick syrup under diminished pressure at 30°. The product was heated at 100° at 15 mm. for 5 hours in order to complete lactonization and then distilled at high vacuum. The main fraction (weight, 1.5 gm.) boiled at 93–95° at 0.05 mm. (bath temperature, 116–118°). It was quite mobile and had N_D^{25} , 1.4572.

Its analysis was as follows:

3.366 mg. substance: 6.284 mg. CO_2 and 2.201 mg. H_2O .

4.564 “ “ : 16.800 “ AgI.

$\text{C}_8\text{H}_{14}\text{O}_6$. Calculated. C 50.50, H 7.4, OMe 48.96

Found. “ 50.91, “ 7.3, “ 48.63

100 mg. substance required 5.36 cc. 0.1 N NaOH.

$\text{C}_8\text{H}_{14}\text{O}_6$. Calculated. 5.26 cc.

The substance displayed the following specific rotations.

$$[\alpha]_D^{27} = \frac{+1.28^\circ \times 100}{2 \times 0.924} = +69.3^\circ \text{ (in chloroform)}$$

$$[\alpha]_D^{27} = \frac{+2.02^\circ \times 100}{2 \times 0.885} = +114.1^\circ \text{ (in dry ether)}$$

$$[\alpha]_D^{27} = \frac{+1.77^\circ \times 100}{2 \times 1.036} = +85.4^\circ \text{ (in benzene)}$$

The rotation of the free acid was determined in the usual manner by forming the sodium salt, adding the equivalent amount of hydrochloric acid, and observing the rotation immediately. Calculated as lactone, the initial specific rotation was as follows:

$$[\alpha]_D^{25} = \frac{+0.44^\circ \times 100}{2 \times 0.647} = +34.0^\circ \text{ (in water)}$$

The course of the hydrolysis of the lactone in aqueous solution was studied polarimetrically. The results obtained when $l = 2$ dm. and $c = 2.3$ are given in Table II.

The lactone would, therefore, appear to belong to “the moderately rapidly hydrolyzing”¹⁰ group of δ -lactones since the time

¹⁰ Carter, S. R., Haworth, W. N., and Robinson, R. A., *J. Chem. Soc.*, 2125 (1930).

required for attainment of half the equilibrium value was estimated to be 14 hours.

Simultaneous Hydrolysis and Oxidation of 2, 3, 4-Trimethyl Methylriboside with Nitric Acid—2 gm. of syrupy distilled trimethyl methylriboside were dissolved in 20 cc. of concentrated nitric acid (sp. gr. 1.42) at 20° and the temperature raised to 60°. At 65° a vigorous reaction took place and the temperature was maintained at 65–70° for 15 minutes. Afterwards the temperature was gradually raised to 95° and maintained there for 7 hours. The reaction mixture was cooled, diluted with distilled water, and evaporated under diminished pressure at 45° with continual additions of water. This procedure was repeated

TABLE II
Conversion of Lactone into Acid

Time	$[\alpha]_D^{25}$	Lactone	Time	$[\alpha]_D^{25}$	Lactone
<i>hrs</i>	<i>degrees</i>	<i>per cent</i>	<i>hrs.</i>	<i>degrees</i>	<i>per cent</i>
0	−4.4	100.0	67	+12.7	55.5
20	+8.4	66.7	115	+14.9	49.7
27	+9.7	63.3	139	+15.6	47.9
44	+10.5	61.2	163	+16.3	46.1
52	+11.9	57.6	191	+17.1	44.0
			241	+17.1	constant

many times until the final distillate was free from nitric acid (2 days). The colorless viscid product was dried and then esterified by boiling it for 7 hours with 50 cc. of methyl alcohol containing 2 per cent of hydrogen chloride. After the mixture had been cooled, and the acid neutralized with dry silver carbonate, the methyl alcohol was removed by evaporation under diminished pressure and the product was distilled at high vacuum. A colorless very mobile liquid was collected at 77–78° at 0.02 mm. (bath temperature, 92–96°). Yield, 1.36 gm. $N_D^{24.5} = 1.4335$.

Its composition was as follows:

3.035 mg. substance: 5.335 mg. CO₂ and 1.975 mg. H₂O.

3.241 " " : 15.390 " AgI.

C₁₀H₁₆O₇. Calculated. C 47.98, H 7.3, OMe 62.02

Found. " 47.94, " 7.3, " 62.68

It was optically inactive in water under conditions ($c = 1.2$, $l = 2$) where a specific rotation of $\pm 1^\circ$ would easily have been detectable. The analytical figures and the optical inactivity showed it to be the dimethyl ester of an inactive trimethoxyglutaric acid.

THE ACTION OF BENZOIC PERACID ON SUBSTITUTED GLUCALS. II

BY P. A. LEVENE AND R. STUART TIPSON

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York)

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In Paper I of this series it was shown¹ that perbenzoic acid combines with triacetylglucal to yield a benzoyl triacetylglucose, but the question of the position of the benzoyl group was left undecided. Simultaneously Tanaka² described similar experiments, and further, he acetylated the benzoyl triacetylglucose, obtaining a benzoyl tetracetylglucose. We wish to point out that his product is identical with that obtained by Zemplén and László,³ in 1915, by the action of silver benzoate upon bromotetracetylglucose. There thus remains little doubt but that in this compound the benzoyl group is situated at position (1) of the sugar chain.

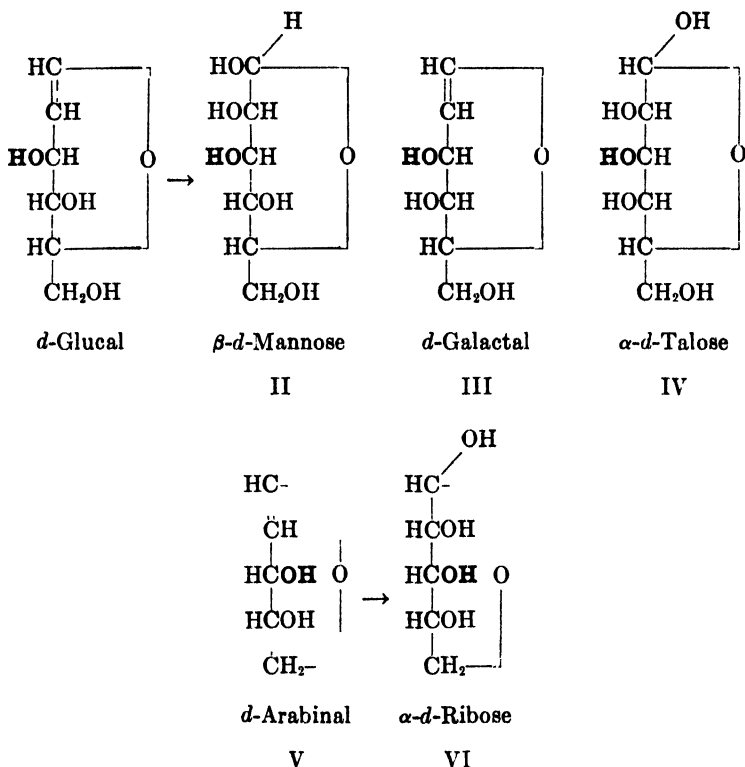
In a similar way it has now been shown that the isolable product obtained by the action of perbenzoic acid on triacetylgalactal is 1-benzoyl-3, 4, 6-triacetylgalactose, that is, that when position (3) is acetylated, the only recognized product is a derivative of galactose. On the other hand, by the action of the same reagent, the non-substituted galactal forms talose predominately.

On comparison of the three examples so far studied of transformation of a simple "glucal" to a sugar, it is noted that a directive influence is apparently exercised by the position of the hydroxyl on carbon atom (3). Thus, from figures I and II and from figures III and IV it can be seen that both in glucal and in galactal the hydroxyl of carbon atom (3) is situated to the *left*, and the two glucals add the hydroxyl on carbon atom (2) to the *left* likewise,

¹ Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **88**, 513 (1930).

² Tanaka, C., *Bull. Chem. Soc. Japan*, **5**, 214 (1930).

³ Zemplén, G., and László, E. D., *Ber. chem. Ges.*, **48**, 915 (1915).



whereas in arabinol (V) the hydroxyl of carbon atom (3) is situated to the *right* and the "glucal" adds a hydroxyl on carbon atom (2) to the *right* likewise. In all three cases hydroxyls (2) and (3) of the preponderating sugar produced are in the *cis* position. Is it not possible that a transitory oxide is formed between carbon atoms (2) and (3), whereas when the hydroxyl of position (3) is blocked, the oxide forms between carbon atoms (1) and (2)?

It must be emphasized that, by the action of perbenzoic acid on glucals, *both* epimeric sugars result but that one largely predominates. The most striking example is the case of galactal. One epimer, however, predominates to such an extent that from the practical view-point the other may be disregarded. A review of the yields recorded in the literature substantiates this statement.

Glucal \rightarrow 68 to 81 per cent yield of mannose.⁴

" \rightarrow 60 " " " " α -methylmannoside.⁵

Cellobial \rightarrow 90 " " " " 4-glucosido- α -mannose.^{5,6}

" \rightarrow 69 " " " " 4-glucosido- α -methylmannoside.⁶

Lactal \rightarrow 70 " " " " 4-galactosido- α -mannose.⁷

d-Arabinal \rightarrow a preponderance of *d*-ribose.⁸

On the other hand, when position (3) of the glucal is methylated or acetylated, the *isolable* product has *trans* groupings at positions (2) and (3). For example:

3-Methylglucal \rightarrow 29.8; 27.5; 46.7 per cent yield of 3-methylglucose actually isolated.¹

Triacetylglucal \rightarrow 30.5; 22.1 per cent of benzoyl triacetylglucose actually isolated^{1,2} and about 20 per cent of a mannose derivative.⁹

Trimethylglucal \rightarrow a preponderance of trimethylglucose.⁹

On the basis of these observations it is possible, then, to predict that idose (idal) will lead to gulose, and that gulose (triacetyl gual) will lead to idose; altrose (altral) will lead to allose, and that allose (triacetyl allal) will lead to altrose; xylose (xylal) will lead to lyxose, and that lyxose (diacetyl lyxal) will lead to xylose. Thus a way is suggested for the preparation and interconversion of the rarer sugars.

EXPERIMENTAL

Preparation of Crystalline Triacetylglactal—120 gm. of zinc dust were added to 1000 cc. of cold 50 per cent aqueous acetic acid with vigorous mechanical stirring at 0°. Finely powdered bromoacetylglactose (m.p., 81°; $[\alpha]_D^{25} = +235.4^\circ$ in benzene) was now added in ten portions of 10 gm. each, during 45 minutes. After all had been added, the reaction mixture was stirred for a further 100 minutes, the temperature being maintained at 0°, after which the mixture was filtered.

⁴ Bergmann, M., and Schotte, H., *Ber. chem. Ges.*, **54**, 440 (1921).

⁵ Bergmann, M., and Schotte, H., *Ber. chem. Ges.*, **54**, 1564 (1921).

⁶ Haworth, W. N., Hirst, E. L., Streight, H. R. L., Thomas, H. A., and Webb, J. I., *J. Chem. Soc.*, 2636 (1930).

⁷ Haworth, W. N., Hirst, E. L., Plant, M. M. T., and Reynolds, R. J. W., *J. Chem. Soc.*, 2644 (1930). Bergmann, M., *Ann. Chem.*, **434**, 79 (1923). Watters, A. J., and Hudson, C. S., *J. Am. Chem. Soc.* **52**, 3472 (1930).

⁸ Gehrke, M., and Aichner, F. X., *Ber. chem. Ges.*, **60**, 918 (1927).

⁹ Hirst, E. L., and Woolvin, C. S., *J. Chem. Soc.*, 1131 (1931).

The filtrate was extracted ten times with 200 cc. portions of toluene, the toluene extract dried over anhydrous sodium sulfate and then evaporated to a thick syrup under diminished pressure at 40°. Two further portions of 200 cc. of toluene were run in and evaporated off. Finally, this syrup was dissolved in 200 cc. of benzene and evaporated to a thick syrup, from which traces of the solvent were removed at high vacuum at 40°. Yield, 58 gm.

As crystallization of this crude product could not be induced it was subjected to distillation at high vacuum. The main fraction (42 gm.) boiled at 134° at 0.01 mm. On cooling it was obtained as a colorless, fairly mobile syrup which crystallized spontaneously after standing at room temperature for 14 days, with occasional scratching with a glass rod. It was recrystallized from a mixture of ether and petroleum ether, crystals 2 cm. in length being readily obtained. The crystals melt at 30° and have a bitter taste. The product had the following composition.

3.465 mg. substance: 6.700 mg. CO₂ and 1.860 mg. H₂O.

C₁₂H₁₆O₇. Calculated. C 52.92, H 5.9
Found. " 52.74, " 6.0

Its rotation was

$$[\alpha]_D^{25} = \frac{-0.62^\circ \times 100}{2 \times 2.506} = -12.4^\circ \text{ (in chloroform)}$$

Action of Perbenzoic Acid on Triacetylglactal—Perbenzoic acid was prepared in good yield by a slight modification of the method of Hibbert and Burt,¹⁰ a solution of sodium methylate in methyl alcohol being found preferable to the ethyl derivative. 25 gm. of crystalline triacetylglactal were dissolved in 50 cc. of dry chloroform and the resultant solution cooled to 0°. A cold solution of 13.97 gm. of perbenzoic acid in 270 cc. of dry chloroform was now added and the mixture kept at 8–10° for 48 hours and then at room temperature for 12 hours. At the end of this time a little more than the theoretical amount of perbenzoic acid had disappeared. The solution was evaporated to dryness under diminished pressure at 25° and extracted repeatedly with boiling petroleum ether (60–70°) under a reflux. The residual insoluble syrup

¹⁰ Hibbert, H., and Burt, C. P., *J. Am. Chem. Soc.*, **47**, 2240 (1925).

(weight, 36 gm.) was dissolved in absolute ethyl alcohol and evaporated to a syrup which crystallized to a solid mass. It was recrystallized from 95 per cent ethyl alcohol. Yield, 12.5 gm. (33.2 per cent of the theoretical yield); m.p., 189°. Its analysis was as follows.

4.660 mg. substance: 9.545 mg. CO₂ and 2.240 mg. H₂O.

C₁₁H₂₂O₁₀. Calculated. C 55.58, H 5.4

Found. " 55.85, " 5.4

Its rotation was

$$[\alpha]_D^{25} = \frac{+ 0.55^\circ \times 100}{2 \times 1.145} = + 24.0^\circ \text{ (in chloroform)}$$

The remaining 23.5 gm. of syrupy product could not be induced to crystallize. It consisted presumably of a mixture of 1-benzoyl triacetylgalactose with the corresponding talose derivative.

Acetylation of 1-Benzoyl-3, 4, 6-Triacetylgalactose—1.0 gm. of benzoyl triacetylgalactose was dissolved in a cold mixture of 10 cc. of pyridine and 10 cc. of acetic anhydride and the resulting solution was kept at 0° for 50 hours.

The reaction mixture was diluted with 200 cc. of chloroform and the solution washed successively with ice-cold dilute sulfuric acid, ice-cold dilute sodium bicarbonate solution, and finally with ice water until neutral. It was dried over anhydrous sodium sulfate and evaporated to a syrup. The product was dissolved in absolute alcohol and again evaporated, whereupon it crystallized spontaneously. Yield, 0.95 gm. On recrystallization from 95 per cent ethyl alcohol it had a melting point of 122°. Its composition was as follows:

4.342 mg. substance: 8.900 mg. CO₂ and 2.135 mg. H₂O.

C₂₁H₃₄O₁₁. Calculated. C 55.73, H 5.3

Found. " 55.89, " 5.5

Its rotation was

$$[\alpha]_D^{25} = \frac{- 0.08^\circ \times 100}{2 \times 1.344} = - 2.98^\circ \text{ (in chloroform)}$$

A mixed melting point determination with an authentic specimen of 1-benzoyl-2, 3, 4, 6-tetracetylgalactose (see next paragraph) showed no depression.

Action of Silver Benzoate upon Bromoacetylgalactose—To a solution of 10 gm. of crystalline bromoacetylgalactose in 50 cc. of toluene were added 7 gm. of dry, finely powdered silver benzoate and the mixture was heated under a reflux at 95° for 20 minutes. It was then shaken with a little charcoal, filtered, and the filtrate (having been found free of bromine) evaporated under diminished pressure at 35° to a very thick syrup. Final traces of solvent were removed at the high vacuum pump at 40°, whereupon the product crystallized immediately to a solid mass. Yield, 10.5 gm. After recrystallization from 95 per cent ethyl alcohol it had a melting point of 121° and its composition was as follows:

5.105 mg. substance: 10.390 mg. CO₂ and 2.435 mg. H₂O.

C₂₁H₂₄O₁₁. Calculated. C 55.73, H 5.3

Found. " 55.51, " 5.3

Its rotation was

$$[\alpha]_D^{25} = \frac{\pm 0.01^\circ \times 100}{2 \times 3.420} = \pm 0.15^\circ \text{ (in chloroform)}$$

Preparation of Crystalline Galactal (Tatal)—To a solution of 70 gm. of barium hydroxide octahydrate in 800 cc. of distilled water were added 30 gm. of crystalline triacetylgalactal and the mixture was shaken on a shaking machine for 15 hours. The excess baryta was now neutralized by passing in gaseous carbon dioxide and the mixture filtered. The filtrate was shaken with a little charcoal, filtered, and evaporated to dryness under diminished pressure at 40°. The last traces of water were removed by running in two portions of absolute alcohol and evaporating to dryness each time.

The mixture of barium acetate and galactal was now extracted three times with boiling absolute alcohol under a reflux, the extract shaken with a little charcoal, filtered, and evaporated under diminished pressure at 30°, the galactal crystallizing towards the end of the distillation. In order to remove traces of ash, the product was dissolved in 300 cc. of absolute alcohol, and 1700 cc. of dry ether were cautiously added with stirring. The flocculent precipitate was removed by filtration on a fluted filter and the resulting clear, colorless filtrate evaporated to dryness under diminished pressure. The galactal was recrystallized from 100 cc. of hot, dry, neutral ethyl acetate, being obtained in rosettes of

fine needles on cooling slowly. Yield, 12 gm.; m.p., 100°. It had the following composition:

5.002 mg. substance: 9.042 mg. CO₂ and 3.102 mg. H₂O.

C₆H₁₀O₄. Calculated. C 49.28, H 6.9

Found. " 49.29, " 6.9

Galactal is intensely sweet, followed by a bitter after-taste. It is not hygroscopic when ash-free and dry. It readily decolorizes dilute bromine water. It does not give a positive test with Schiff's reagent and does not reduce boiling Fehling's solution. It is extremely sensitive to concentrated acids giving a dark brown precipitate, but is quite stable in alkaline solution. It is readily soluble in water, absolute alcohol, methyl alcohol, and warm ethyl acetate; much less soluble in ether and almost insoluble in benzene and petroleum ether. The presence of a slight trace of ash lowers the melting point considerably.

For obtaining galactal, a much less tedious method (a modification of that of Isbell¹¹) was later used, the product being obtained in almost quantitative yield.

40 gm. of crystalline triacetylgalactal were dissolved in 1 liter of absolute methyl alcohol and the solution cooled to 0°. 30 cc. of a 0.25 molal solution of barium methylate in absolute methyl alcohol were added and the solution kept at 0° for 24 hours.

The solution was then saturated in the cold with carbon dioxide and 200 cc. of distilled water were added. The solution was again saturated with carbon dioxide and was then heated until gently boiling. A little charcoal was added, the mixture filtered, and the filtrate evaporated to dryness under diminished pressure at 30°. The crystalline product was dissolved in absolute ethyl alcohol and was again evaporated to dryness in order to remove traces of water.

The galactal was freed from traces of ash as previously indicated, and was then recrystallized from dry ethyl acetate. Yield, 21 gm.; m.p., 100°. Glucal is readily obtained crystalline by hydrolyzing triacetylglucal in the same manner.

Action of Perbenzoic Acid upon Galactal (Talal)—A solution of 7 gm. of galactal in 70 cc. of distilled water was cooled until ice formed, and then a cold solution of 7.7 gm. of perbenzoic acid in

¹¹ Isbell, H. S., *Bureau Standards J. Research*, **5**, 1179 (1930).

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98 cc. of ethyl acetate was added. The mixture was shaken at 8–10° for 2 hours, after which the ethyl acetate layer still contained perbenzoic acid, but the aqueous layer had lost its power of decolorizing very dilute bromine water. The two layers were separated, the ethyl acetate layer being twice shaken with a little water, and these aqueous extracts added to the main aqueous solution. The combined aqueous solution was now extracted three times with benzene, or until the benzene extract no longer gave a pink color with acidified potassium iodide solution, and then traces of benzene were removed under diminished pressure. It was diluted to 250 cc. and the aldohexose content of aliquot portions determined by the method of Willstätter and Schudel¹² as modified by Goebel¹³ (see Table I).

2.5 cc. of sugar solution consumed 8.95 cc. 0.1 N iodine solution.

∴ yield of aldohexose = 8.06 gm. (93.4 per cent of theory).
The specific rotation of this solution, calculated from the above aldohexose concentration, was

$$[\alpha]_D^{25} = \frac{+ 2.71^\circ \times 100}{2 \times 3.224} = + 42.0^\circ \text{ (in water)}$$

This solution was now evaporated to a thick syrup under diminished pressure at room temperature. It was dissolved in absolute ethyl alcohol and evaporated to a thick syrup, the process being repeated several times more with ethyl alcohol and benzene. It was dissolved in ethyl alcohol and allowed to stand overnight at room temperature. The crystalline powder which had separated was filtered off (Fraction 1).

A second fraction (Fraction 2) was next obtained and on concentrating the mother liquor to a thick syrup in a vacuum desiccator over phosphorus pentoxide, and then triturating with methyl alcohol, this also crystallized spontaneously (Fraction 3).

Fraction 1—M.p. crude, 160°; weight, 0.26 gm. It had the following composition.

5.125 mg. substance: 7.535 mg. CO₂ and 3.035 mg. H₂O.

C₆H₁₂O₆. Calculated. C 39.98, H 6.7

Found. " 40.09, " 6.6

¹² Willstätter, R., and Schudel, G., *Ber. chem. Ges.*, **51**, 780 (1918).

¹³ Goebel, W. F., *J. Biol. Chem.*, **72**, 801 (1927).

Its rotation in water was

$$[\alpha]_D^{25} = \frac{+ 0.89^\circ \times 100}{1 \times 1.104} = + 80.6^\circ \text{ (final)}$$

Fraction 2—M.p., 146°; weight, 0.41 gm. Its rotation in water was

$$[\alpha]_D^{25} = \frac{+ 1.43^\circ \times 100}{1 \times 1.092} = + 131.0^\circ \text{ (initial)}$$

$$[\alpha]_D^{25} = \frac{+ 0.75^\circ \times 100}{1 \times 1.092} = + 68.7^\circ \text{ (final)}$$

Fraction 3—M.p., 129–130°; sweet taste; weight, 3.12 gm. It had the following composition.

4.971 mg. substance: 7.417 mg. CO₂ and 3.045 mg. H₂O.

C₆H₁₂O₆. Calculated. C 39.98, H 6.7

Found. " 40.69, " 6.9

0.0648 gm. consumed 7.12 cc. 0.1 N iodine.

C₆H₁₂O₆·CHO. Calculated, 7.20 cc. 0.1 N iodine

Its rotation in water was

$$[\alpha]_D^{25} = \frac{+ 0.66^\circ \times 100}{1 \times 1.052} = + 62.7^\circ \text{ (initial)}$$

$$[\alpha]_D^{25} = \frac{+ 0.34^\circ \times 100}{1 \times 1.052} = + 32.3^\circ \text{ (final)}$$

Preparation of Various Substituted Phenylhydrazones

Fraction 1—To 0.2014 gm. of crystalline material in a small evaporating dish was added a solution of 0.2091 gm. of recrystallized *p*-bromophenylhydrazine dissolved in about 20 cc. of absolute methyl alcohol. The mixture was warmed gently until all the sugar had dissolved and was then evaporated to dryness. On being scratched with a glass rod, it crystallized to a solid mass. It was recrystallized from 5 per cent aqueous ethyl alcohol, being obtained as colorless feathery needles of m.p. 168–170. After a second recrystallization from 5 per cent ethyl alcohol, the melting point was unchanged. A mixed melting point determination with

authentic galactose *p*-bromophenylhydrazone (m.p., 168–169°) gave m.p., 168–169°. Its composition was as follows:

3.800 mg. substance:	5.790 mg. CO ₂ and 1.695 mg. H ₂ O.
4.670 " "	: 0.320 cc. N ₂ (756 mm. at 23.5°).
4.678 " "	: 2.512 mg. AgBr.
C ₁₂ H ₁₇ O ₅ N ₂ Br. Calculated.	C 41.25, H 4.9, N 8.03, Br 22.89
Found.	" 41.55, " 5.0, " 7.85, " 22.85

Fraction 3—An attempt was made to prepare the *p*-bromophenylhydrazone under the conditions outlined above. After evaporation to dryness and trituration with a little methyl alcohol, the substance crystallized. After trituration with 5 per cent ethyl alcohol and filtration, the crude, dry product had a melting point of 146–148°. On being dissolved in 10 cc. of 5 per cent ethyl alcohol it would not crystallize on cooling, or nucleation. It was therefore evaporated slowly in a vacuum desiccator over phosphorus pentoxide, whereupon it crystallized completely, but was not further investigated on account of its great solubility.

Methylphenylhydrazone—0.5000 gm. of crystalline substance (*Fraction 3*) was treated with a solution of 0.3388 gm. of methylphenylhydrazine in 20 cc. of methyl alcohol. The solution was evaporated to dryness whereupon it crystallized to a solid mass. The crude product had a melting point of 146°. After one recrystallization from methyl alcohol, the melting point was 152°; after a second recrystallization from methyl alcohol it was 154°. The mixed melting point determination with authentic galactose methylphenylhydrazone (m.p., 185–186°) was 165–170°. The substance had the following composition.

4.237 mg. substance:	8.505 mg. CO ₂ and 2.730 mg. H ₂ O.
4.065 " "	: 0.352 cc. N ₂ (756 mm. at 26.0°).
C ₁₃ H ₂₀ O ₅ N ₂ . Calculated.	C 54.90, H 7.1, N 9.86
Found.	" 54.75, " 7.2, " 9.83

Blanksma and van Ekenstein¹⁴ found that talose methylphenylhydrazone had a melting point of 154° whereas von Braun and Bayer¹⁵ give its melting point as 220–222° and in addition the lat-

¹⁴ Blanksma, J. J., and van Ekenstein, W. A., *Chem. Weekblad*, **5**, 771 (1908).

¹⁵ von Braun, J., and Bayer, O., *Ber. chem. Ges.*, **58**, 2215 (1925).

ter investigators state that the *p*-bromophenylhydrazone separates quantitatively from 5 per cent alcoholic solution and has a melting point of 205°.

Oxidation with Nitric Acid

0.250 gm. of the crystalline sugar (Fraction 1) was dissolved in 5 cc. of 50 per cent nitric acid (by volume) and the solution kept at room temperature overnight. The solution turned pale green in color and crystals started to separate out. After standing for several days the nitric acid was removed by repeated evaporation with water. The crystalline product was triturated with dry acetone, dried, and weighed. Yield of mucic acid, 0.184 gm.; m.p., 216° with frothing and decomposition.

TABLE I
Yields of Aldohexose in Various Experiments

Amount of galactal used	0.1 N iodine consumed by 2.5 cc. of aldohexose solution	Total volume of aldohexose solution	Yield of aldohexose	α_D , 2 dm. tube	$[\alpha]_D$
gm.	cc.	cc.	per cent	degrees	degrees
7.0	8.95	250	93.4	+2.71	+42.0
3.0	9.75	100	94.9	+2.96	+42.2
6.8	9.14	250	98.1	+3.06	+46.5
5.0	5.99	250	87.4	+1.80	+42.0
5.0	6.05	250	88.5	+1.89	+43.4
5.0	5.99	250	87.5	+1.92	+44.6

Under similar conditions no crystalline material was isolated from the "talose" (Fraction 3).

Partial Purification of Talose

From these experiments it appears that Fraction 1 consisted of practically pure galactose, whereas Fraction 3 undoubtedly contained a large quantity of a less dextrorotatory sugar.

In further preparative experiments, details of which are recorded in Table I, the product obtained by the action of perbenzoic acid was isolated as a thick syrup (after removal of traces of perbenzoic acid by extraction with benzene) and after evaporation once with absolute ethyl alcohol the "talose" fraction (m.p., 129°) separated first, without recourse being had to nucleation. That this was not

TABLE II
Purification of Crude Talose

Weight of crude talose, m p. 129°	Experiment 1 2.71 gm.	Experiment 2 3.50 gm.
Extraction with 80 per cent alcohol Extract 1; overnight at 25°	7 cc.; $\alpha_D^{25} = +9.12^\circ$ (initial)	10 cc.; $\alpha_D^{25} = +7.79^\circ$ (initial) $\alpha_D^{25} = +7.52^\circ$ (final)
Extract 2; 5 min. at 25°	10 cc.; $\alpha_D^{25} = +4.90^\circ$ (final)	10 cc.; $\alpha_D^{25} = +7.10^\circ$ (initial) $\alpha_D^{25} = +5.70^\circ$ (final)
Extract 3; 5 min. at 25°	10 cc.; $\alpha_D^{25} = +3.42^\circ$ (final)	10 cc.; $\alpha_D^{25} = +4.77^\circ$ (initial) $\alpha_D^{25} = +3.96^\circ$ (final)
Yields		
A. Insoluble residue	0.33 gm. (m.p., 162-163°)	0.45 gm. (m.p., 169-170°)
B. Soluble, crystalline material from extracts (a) Containing no ammonia (b) Containing ammonia (Ex- periments 1 and 2 com- bined)	1.72 gm.	1.92 gm.
	1.78 gm.	

a pure specimen of talose, but contained traces of galactose, was revealed by extraction with 80 per cent ethyl alcohol (by volume). The details of two typical extraction experiments are given in Table II. The rotations were measured in a 2 dm. tube, the initial reading being made immediately after filtering a portion of the supernatant liquor, and the final reading being observed after addition of one drop of concentrated ammonia.

The insoluble sugar residue from Experiment 1 proved to be almost pure α -galactose¹⁶ since its rotation in 80 per cent ethyl alcohol was

$$[\alpha]_D^{25} = \frac{+ 0.28^\circ \times 100}{2 \times 0.112} = + 125.0^\circ \text{ (initial)}$$

$$[\alpha]_D^{25} = \frac{+ 0.17^\circ \times 100}{2 \times 0.112} = + 75.9^\circ \text{ (final)}$$

The crude "talose" (m.p., 129°) used as starting material in Experiment 2 had the following rotation in 80 per cent ethyl alcohol.

$$[\alpha]_D^{25} = \frac{+ 0.17^\circ \times 100}{2 \times 0.108} = + 78.7^\circ \text{ (initial)}$$

$$[\alpha]_D^{25} = \frac{+ 0.10^\circ \times 100}{2 \times 0.108} = + 46.3^\circ \text{ (final)}$$

The crystalline ammonia-free product from these two experiments was combined and this once purified talose (3.64 gm.) submitted to a second purification in the same manner. It was first shaken for 2 hours with 10 cc. of 80 per cent alcohol at 27° and the filtrate evaporated slowly to give a crystalline powder (1.52 gm.). The insoluble sugar from the first extraction was then extracted for 5 minutes with 10 cc. of 80 per cent alcohol at 27°. On evaporation, the second extract gave 1.09 gm. of beautifully crystalline material. The insoluble residue, after drying, weighed 0.83 gm. It had a melting point of 130–135° and its rotation in water was

$$[\alpha]_D^{25} = \frac{+ 0.66^\circ \times 100}{2 \times 1.140} = + 29.0^\circ \text{ (initial)}$$

¹⁶ Hudson, C. S., and Yanovsky, E., *J. Am. Chem. Soc.*, **39**, 1013 (1917).

$$[\alpha]_D^{25} = \frac{+ 0.45^\circ \times 100}{2 \times 1.140} = + 19.7^\circ \text{ (final)}$$

Since the solubility of α -galactose¹⁶ in 80 per cent alcohol is 0.27 gm. per 100 cc. of solution (initial) and 0.65 gm. per 100 cc. of solution (final), it seems that talose is probably at least 40 times more soluble initially than α -galactose in 80 per cent alcohol.

At this time we cannot make a final statement of the properties of talose, but work on the preparation and purification of the substance is in progress. 2-Desoxygalactose has also been prepared. Its properties will be reported later.

THE INTERFEROMETRIC DETERMINATION OF ALCOHOL IN BLOOD

BY JOSEPH C. BOCK

(From the Department of Physiological Chemistry, Marquette University
Medical School, Milwaukee)

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INTRODUCTION

A critical study of the methods for the determination of ethanol in biological fluids and tissues brings the realization of the fact that the very large majority of these procedures is not sufficiently accurate to allow the determination of minute amounts nor the estimation of small variations when larger amounts occur.

With the exception of one method by Widmark (1), all procedures remove the alcohol by distillation. The material is diluted with water and the alcohol is distilled *in vacuo*, with steam or under normal pressure.

The alcohol in the distillate is determined by various processes. A large number of these consist of modifications of Nicloux's (2) method. All of the methods in this group are based on the oxidation with bichromate. The reagents used in these methods are not stable and must be restandardized frequently. The color changes, which determine the end-point, are very difficult to observe, the exactness of the procedure being based on the subjective judgement of the observer, to the extent that errors of 10 drops or more may occur. In one of the best of the oxidation methods by Gettler and Tiber (3), the authors themselves admit consistently low results and apply a correction factor.

Fischer and Schmidt (4) convert the alcohol into ethyl nitrite and acetic acid. The nitrite is aerated with a stream of carbon dioxide into a potassium iodide solution acidified with hydrochloric acid. The method is not accurate, is time-consuming, and necessitates the use of complicated apparatus.

In the methods of Bugarsky (5), Stolz (6), and Spechter (7),

bromine is used as an oxidizing agent, the alcohol being converted to acetic acid with the formation of hydrobromic acid. The necessary excess of bromine must be removed by heating, a procedure which results in appreciable losses of hydrobromic acid.

The alcohol in the distillate can be determined by the pycnometer as described by Kühn (8) and Vollmering (9). The procedures as well as the vaporimeter method of Nungesser (10) necessitate several distillations and are of doubtful value where small amounts of alcohol are concerned.

The immersion refractometer is not sufficiently accurate when used with very dilute alcohol solutions.

Although the study of the literature undertaken for this work is not claimed to be exhaustive, we have found only one attempt to remove the proteins before distillation. This method of Maignon (11), however, is rather complicated and inaccurate. In the present work we remove the blood proteins with a single reagent, distil in a very simple distilling apparatus, and determine the alcohol by means of an interferometer. This instrument has been used for this purpose by Kionka (12). He distils the alcohol from the whole blood *in vacuo*, using a very complicated, costly, and fragile apparatus, which shortcomings the present method attempts to overcome.

Interferometric Measurements

Before describing our procedure it is deemed necessary to give a very brief discussion of interferometric measurements. A detailed description of the instrument and its many uses can be obtained from the makers, Carl Zeiss, Jena, Germany. The principles of physics involved in its construction are described by the inventor, Löwe (13, 14).

The Löwe-Zeiss interferometer for liquids (and gasses) is an instrument which measures the *difference* of the refractive indices of two liquids or solutions. Two coherent pencils of light, coming from a small lamp, traverse the two media under examination. If both liquids are of exactly the same density the observer sees through the eyepiece of the instrument two horizontal fields with series of vertical bands, the interference bands. The fields are traversed by two black median bars bordered on each side by colored bands. The two pairs of black bands, the minima of the

first order, coincide only if both liquids are alike. If there is the slightest difference in their refractive indices, the two black bands of the upper field move from their median position, to the right or left. By turning the compensator with the micrometer screw, the bands of the upper field are made to coincide with those of the lower and immovable comparison spectrum. The amount of rotation can be read from the scales attached to the micrometer

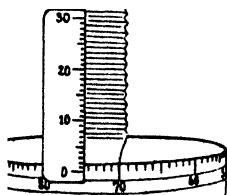


FIG. 1

FIG. 1. Micrometer screw showing the two scales to be considered in the interferometric measurements. The circular or drum scale has 100 divisions; the vertical scale 30.

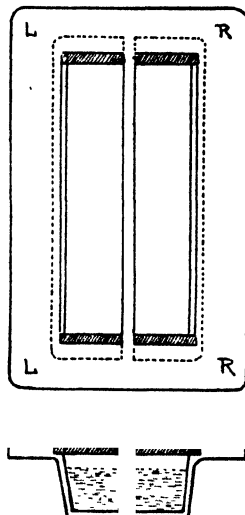


FIG. 2

FIG. 2. The gold-plated metal chamber, consisting of two cells closed with glass at each end, in which the liquids to be compared are contained.

screw. As seen in Fig. 1 there are two scales to be considered. The circular or drum scale has 100 divisions, the vertical scale has 30. One full turn of the drum moves the circular scale 1 division upward on the vertical scale, therefore 1 division of the latter is equivalent to 100 drum scale divisions. The reading shown in Fig. 1 is 174.5.

The liquids to be compared are contained in a gold-plated metal chamber, consisting of two cells closed with glass on each end (Fig. 2). The inside measurements, in the optical axis, vary; 5

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mm., 10 mm., 20 mm., 40 mm., and 80 mm. chambers being available. For the present work the two largest sizes were used. If both cells are filled with the *same* liquid the reading for the *same* chamber will be the same. To illustrate:

40 Mm. Chamber

Both cells water.....	drum reading, 18
" " 0.1 per cent alcohol.....	" " 18
" " 0.01 " " "	" " 18

With the 80 mm. chamber with the same three liquids three readings of 33 are obtained.

When beginning a series of determinations one must always determine the zero value of the chamber as illustrated above. For our purpose water is indicated as comparison fluid. The temperature must be the same throughout the whole set of observations. After the zero value of the chamber has been ascertained, the comparison fluid (water) is left in one cell, preferably the right one. The other cell is emptied by means of a pipette. The tip of the pipette should be protected with a short piece of small rubber tubing, to prevent scratching. The cell is then dried with rolls of filter paper and finally with a wad of absorbent cotton. Soft linen, commonly used in cleaning optical instruments, should be avoided, because it may contain traces of starch which will introduce a serious error. After the cell is dry, it is filled with the alcohol solution and the chamber put in the interferometer. After the temperatures of the cells are equal (3 to 5 minutes) the position of the bands is observed, the micrometer screw is turned until the bands coincide, and the scales are read. The reading is interpreted by means of a previously determined curve (Figs. 3 and 4). The curve is obtained by plotting the scale reading of solutions of known strength against the percentage of these solutions. It is best to plot two or three curves, depending on the kind of work. We have one for concentrations from 0 to 0.01 per cent, the second to 0.1 per cent; a third can be carried as high as the expected results demand. Instead of a curve, interpolation may be used. If, for instance, a known alcohol dilution of 0.02 per cent reads 79 against water in an 80 mm. chamber and another of 0.04 per cent reads 119, a scale reading of 99 would indicate an alcohol concen-

tration of 0.03 per cent. From the above figures it can be easily seen that 1 scale division is equivalent to 0.0005 per cent alcohol when the 80 mm. chamber is used.

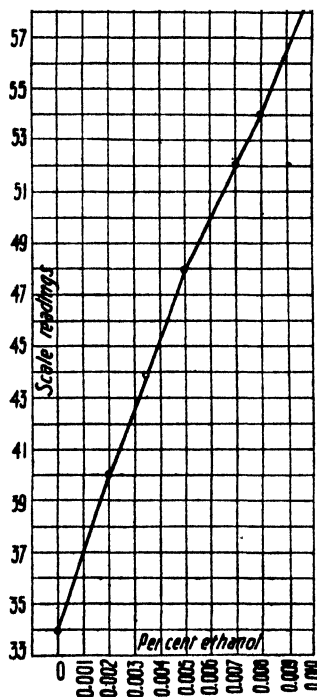


FIG. 3

Fig. 3. Standardization curve; 0.001 to 0.01 per cent

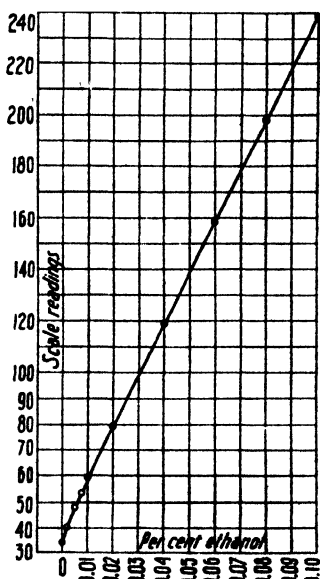


FIG. 4

Fig. 4. Standardization curve; 0.01 to 0.1 per cent

Distilling Apparatus

The purpose of the first set of experiments was to determine to what degree the recovery of very small amounts of alcohol could be accomplished by a simple distillation process.

The distilling flask used is a 50 ml., round bottom, Pyrex flask. The connection leading from the flask to the condenser is made from a 5 ml. pipette having a tube of comparatively large diameter. This tube must be of the same dimension as the condenser tube,

i.e. 6 to 7 mm. or $\frac{1}{4}$ inch outside diameter. The two tubes must touch very closely and are held together by a heavy walled rubber tube. It was found that the condenser tube had to be fairly short, which fact caused a comparatively small condensing surface. In order to get satisfactory recoveries the distillation had to be exceedingly slow. This difficulty was finally overcome by using a metal condenser tube. Either silver¹ or copper¹ tubing may be used, although the use of silver is indicated when the highest accuracy is desired. The length of tube, before bending, is 48 cm. The metal tubing must first be washed with dilute nitric acid and then rinsed with water several times. After steaming the tube, it is washed again two or three times with distilled water. It is advisable to keep the metal condenser tube closed or filled with water when not in use. If the condenser has not been used for more than 24 hours, it is best not only to rinse the tube but to clean it further by distilling about 20 to 30 ml. of water through it. These precautions will not appear superfluous when one considers that the interferometer is capable of measuring refraction differences of liquids within 2 units of the seventh decimal.

The receiver, usually a 25 ml. volumetric flask, contains a small (1 to 2 ml.) amount of water and is placed so that the condenser tube touches the water at the bottom of the flask, forming a seal. As the distillate accumulates, the receiver is gradually lowered, so that the end of the condenser tube is only a short (3 to 5 mm.) distance under the surface of the liquid. The lowering of the flask is easily done by using a wedge-shaped support. The small flame of the micro burner should be shielded as a further precaution against back suction.

Recovery of Alcohol by Distillation

The effectiveness of the distillation procedure was tested in the following manner. A series of alcohol dilutions was made from pure ethanol, certified burettes and flasks being used. The solutions were further checked by means of an immersion refractometer whenever the dilution permitted. 25 ml. quantities were measured into the distilling flask and distilled, a 25 ml. volumetric flask being

¹ The silver tubing can be obtained from the American Platinum Works, Newark, New Jersey. The copper tubing, $\frac{1}{4}$ inch outside diameter, can be bought from any automobile supply store.

used as a receiver. The process was continued until about 20 ml. had been collected. The condenser is rinsed into the flask with small amounts of water, the condenser tube being then above the liquid level. The flask is made to volume and the contents thoroughly mixed. The distillates are analyzed with the interferometer. In this series of experiments we did not use water as comparison fluid, nor did we make use of the plotted curves. We use the original alcohol solutions instead; that is to say, one portion was distilled, the other serves as comparison liquid. It has been mentioned above, that if both cells of the interferometer

TABLE I
Recovery of Alcohol from Solutions of Known Strength

Alcohol		Scale reading†	
Left cell	Right cell	40 mm. chamber	80 mm. chamber
<i>per cent</i>	<i>per cent</i>		
Stock solution..... .0 5	Stock solution.....0.5	17	33
Distillate.0.5	“ “0.5	16	33
“0.5	“ “0 5	17	34
Stock solution . . . 0.5	“ “0.1	17	34
Distillate.0 5	“ “0.1	17	33
Stock solution . . .0 05	“ “0.05	18	35
Distillate.. . . .0.05	“ “0.05	19	35
Stock solution... .0.01	“ “0.01	18	34
Distillate0 01	“ “0 01	17	33

chamber are filled with the same liquid the reading is the zero value. We established this value by using the various alcohol solutions in both cells, and then reading the distillate from each dilution against the original (stock) solution. This procedure furnishes the most accurate check on the method. The instrument can be read accurately within 1 or 2 scale divisions or, in the present case, within limits of 0.0005 to 0.001 per cent ethanol by volume. Table I gives some of the results. From the table it will be seen that all the readings are within 1 scale division of the zero value and no appreciable loss of alcohol has occurred in the distillation process.

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Reagent

Almost every method for the determination of alcohol in blood uses distillation without removing the proteins. The disadvantages of such a procedure are obvious. We found that the blood proteins may be removed and the filtrate used for distillation without any appreciable loss of alcohol. A number of protein precipitants were considered and tried. Those containing volatile material are *ipso facto* excluded. Others did not yield enough filtrate for the determination or called for dilutions too great for our purpose.

A phosphomolybdic acid reagent was found satisfactory. It gives a coarse precipitate and ample filtrate. The composition is as follows: 12 gm. of phosphomolybdic acid and 10 gm. of sodium sulfate (crystals) are dissolved in about 600 ml. of water. 9 ml. of concentrated sulfuric acid are added and the solution is then boiled for approximately 15 minutes. After cooling make to 1 liter.

Procedure

10 ml. of blood are run slowly into a 50 ml., glass-stoppered volumetric flask, containing about 35 ml. of the phosphomolybdic acid reagent. The flask is gently agitated (not shaken) to mix the contents and filled to the mark with reagent. Mix well, let stand about 10 minutes, and transfer to a 50 ml. centrifuge tube. This tube has a slightly constricted neck and is closed with a rubber cap.² The tube is centrifugated for approximately 10 minutes at high speed. The supernatant liquid is poured through a small coarse filter to free it of the small particles of precipitate which adhere frequently to the upper part of the tube.

25 ml. of filtrate are transferred to the distilling flask and distilled at a moderate rate of speed until about 20 ml. have been received in a 25 ml. volumetric flask. The flask is made to volume, the contents are well mixed, and the alcohol is determined in the interferometer.

Tests for Losses in Manipulation

In order to determine if any measurable losses of alcohol will occur with the procedure described above we performed the fol-

² International Equipment Company, Boston, Massachusetts, catalogue; tube No. 520, cap No. 584.

lowing experiments. Instead of using blood we used alcohol solutions of known strength. 10 ml. samples of these solutions were diluted with reagent to 50 ml. and centrifugated in the capped tubes for the same length of time as the blood samples. They were then run through a filter and distilled. In the regular analysis of blood we use water as comparison fluid in the interferometer chamber. In this set of experiments we used the same alcohol dilutions as

TABLE II
Recovery of Added Alcohol

Sample No.	Alcohol added	Alcohol recovered	Difference
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	0.04	0.0385	-0.0015
	0.04	0.0395	-0.0005
	0.04	0.0400	0.0000
2	0.06	0.0605	+0.0005
	0.06	0.0585	-0.0015
	0.06	0.0600	0.0000
	0.06	0.0595	-0.0005
3	0.02	0.0205	+0.0005
	0.02	0.0190	-0.0010
	0.02	0.0205	+0.0005
4	0.1	0.0990	-0.0010
	0.1	0.1000	0.0000
	0.1	0.0985	-0.0015
5	0.2	0.1985	-0.0015
	0.2	0.2000	0.0000
	0.2	0.1995	-0.0005

comparison fluid which we used for the manipulations described above. If we dilute 10 ml. of 0.2 per cent alcohol to 50 ml. in the above procedure we obtain a 0.04 per cent solution. This solution, after centrifugation, filtration, and distillation was compared in the interferometer with a 0.04 per cent alcohol which in turn was obtained by diluting 10 ml. of the same 0.2 per cent alcohol to 50 ml. Several series of triplicate determinations were made with alcohol concentrations varying from 0.01 to 0.2 per cent. The greatest

variation from the theoretical reading, the zero value, was 4 scale divisions, but 93 per cent of the results showed readings within +1 or -1 of the zero value, 2 scale divisions being equivalent to 0.001 per cent of ethanol when the 80 mm. chamber was used.

Recovery of Alcohol Added to Blood

The next problem was to determine whether or not a definite amount of alcohol added to the blood could be successfully recovered. The alcohol contents of bloods were first determined. Accurately measured amounts of alcohol were then added to portions of the same blood and the samples analyzed as described before. From Table II it will be seen that the losses are very small. The results given in Table II are representative of the findings from a large number of determinations.

Interfering Substances

The presence of volatile substances other than ethanol has to be considered. Acetone, acetaldehyde, lactic acid, and glycerol have been reported in human blood. Our distillates were examined for these substances.

Acetone and Acetaldehyde—Traces of these substances were found to be present in less than 3 per cent of the cases studied. The distillates showing positive results were not read in the interferometer.

Glycerol—Dilute solutions of glycerol were distilled in the same manner as the blood filtrates. The results were negative.

Lactic Acid—A 0.1 per cent solution of lactic acid subjected to the usual distillation procedure gave distillates which read within the limits of error of the method.

The present work has confined itself to bloods of normal individuals. It was found that the blood of adults, taken before breakfast, about 12 hours after the last meal, fluctuates from 0.0015 per cent to 0.0113 per cent, the majority of cases varying only from 0.003 to 0.005 per cent ethanol.

A study of the alcohol concentration of pathological bloods and of tissues is in progress.

SUMMARY

A rapid and accurate method for the determination of alcohol in normal blood is presented.

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THE EFFECT OF ARTIFICIAL DRYING UPON THE VITAMIN A CONTENT OF ALFALFA*

By SIGFRED M. HAUGE AND WILLIAM AITKENHEAD

*(From the Departments of Research Chemistry and Agricultural Engineering,
Agricultural Experiment Station, Purdue University, Lafayette)*

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The recognition of the highly nutritive value of alfalfa has contributed to its extensive use in the rations of poultry and farm animals. Vitamin A is present in relatively large amounts and is probably the most important single nutritive factor in alfalfa that may be affected in the curing.

The association of vitamin A potency and greenness in various plant tissues has been emphasized by a number of investigations (1-3). Coward (4) found that vitamin A is completely destroyed when the leaf dries up and dies. Drummond and his coworkers (5) reported that samples of hay may show very different vitamin A values, and stated, "We think it very probable therefore, that good quality meadow hays which have been prepared under conditions which preserve to some extent the green colour of the constituent plants, may be most valuable foodstuffs for cows during winter feeding and superior to the very dry brown hays." Likewise, Dutcher (6) stated, "Practical feeders have observed that bright green alfalfa, properly dried and cured, is superior to the light colored crops, bleached out by unsatisfactory climatic conditions or careless agricultural practice. Careful investigations of the vitamin content of the leafy hays produced under various conditions of drying and curing should yield information of scientific and practical importance."

In studying the vitamin A content of alfalfa, Bethke and Kick (7) found that exposing the alfalfa hay to sun, rain, or dew over a period of several days resulted in marked loss of vitamin A. More

* Published with the approval of the Director of the Agricultural Experiment Station, Purdue University.

recently, Russell (8) in his studies on the effect of the curing process on the vitamin A content of alfalfa leaves found that leaves which had been dried by artificial heat retained their green color and contained at least 7 times as much vitamin A as the leaves from hay which had been cured in the field so that much of its green color was lost.

With the development of processes for the artificial drying of agricultural products, arises the possibility that such processes may be used extensively in the future and that the products so dried may become of economic importance. It therefore seemed timely to study the effect of such processes on the vitamin A content of alfalfa.

TABLE I

Rations Used to Test Vitamin A in the Various Preparations of Alfalfa

Ingredients	Experiment I		Experiments II and III	
	Vitamin A -deficient diet	Test diets	Vitamin A -deficient diet	Test diets
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Alfalfa.....	0	X*	0	X*
Dextrin.....	38	38-X	36	36-X
Ground white corn....	25	25	25	25
Casein.....	18	18	18	18
Lard	10	10	10	10
Yeast.....	3	3	5	5
Salt Mixture 185†....	4	4	4	4
Agar-agar.....	2	2	2	2

* The proportions of alfalfa were varied for the different lots.

† McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).

EXPERIMENTAL

For the determination of the vitamin A content of the various samples of alfalfa used in these studies, the curative method was used. Young albino rats were employed as the experimental animals. The number of animals employed in each lot ranged from four to eight animals, the sex distribution being about equal. Each rat was placed in an individual cage of a type which prevented access to excreta. The food was given *ad libitum* in modified McCollum type feeding cups. The composition of the vari-

ous rations is indicated in Table I. The alfalfa in all lots was from either second or third cuttings. Ultra-violet light was used to protect the animals against rickets. During the preliminary depletion period, the rats were restricted to the vitamin A-deficient diets until the storage of vitamin A in the animals was exhausted, as evidenced by definite symptoms of vitamin A deficiency. At this point, usually 3 to 4 weeks, the animals were transferred to the respective test diets. Weekly records of weight of the animals and food consumption were kept.

Experiment I—In this first experiment, comparisons were made of the vitamin A content of samples of alfalfa cured by the ordinary field method and dried by mechanical dryers. Since the

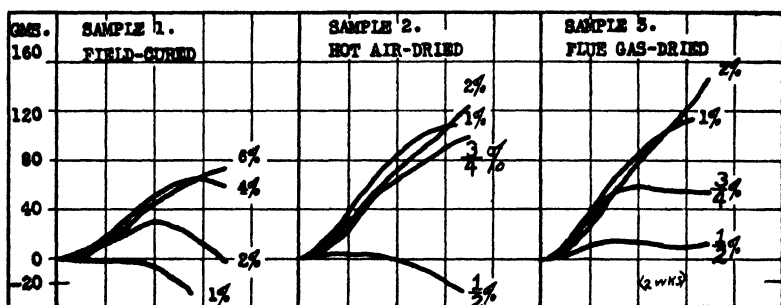


CHART 1. Composite growth curves of groups of rats showing the relative vitamin A content of field-cured and artificially dried alfalfa.

fundamental principles of the artificial drying processes may be divided into two classes, first, drying with heated air, and second, drying with hot flue gas, the materials selected for these studies were limited to samples dried by processes involving either heated air or hot flue gas. In addition to these two samples, a sample of alfalfa hay which had been sun-dried in the field was taken for comparison. These samples were secured in 1929 and tested the following winter.

Sample 1—This sample of alfalfa was sun-dried by the usual field curing process. Although the material showed definite bleaching of the green color, being light green in color, it would be classified as a fine product of field-cured alfalfa.

Sample 2—This sample of alfalfa was dried by means of artifi-

cially heated air. The drying period was about half an hour. The resulting product was somewhat darker green than the fresh alfalfa.

Sample 3—This sample of alfalfa was dried very rapidly with flue gas of high initial temperature. To eliminate as far as possible the entrance of air into the machine during the introduction of the material, the green alfalfa was introduced through a rotary valve. Thus the drying was accomplished in the presence of the minimum amount of air. The dried product was somewhat darker green than Sample 2.

In testing these samples, alfalfa was fed at levels ranging from 0.5 per cent to 6 per cent of the diet. The results of these tests are shown in Chart 1. In comparing the two samples of the artificially dried alfalfa, it is seen that there is practically no difference in their vitamin A content, and that high heat apparently had not adversely affected the vitamin. However, in the case of the field-cured sample, it may be seen that considerable destruction of the vitamin has taken place during the drying process. In fact, the vitamin A content of the machine-dried samples is many times as great as that of the field-cured alfalfa.

Experiment II—Although the first experiment showed that the artificial drying processes were superior to the field curing method in preserving the vitamin in alfalfa, it did not disclose the factors responsible for the preservation or destruction. Therefore, this experiment was planned to gain an insight into this problem.

The materials used for this test were obtained from alfalfa clippings, 8 to 10 inches high. The alfalfa was cut early in the morning in July, 1930, and quartered and treated as soon as possible by the following methods.

Sample 4—The alfalfa was placed on screens and exposed in an open window to the direct rays of the sun for about 16 hours until dry.

Sample 5—This portion of alfalfa was treated in an autoclave in the presence of live steam at 17 pounds of pressure for 1 hour and then dried by exposure to the sun, similarly to Sample 4. This sample on autoclaving lost its bright green color and assumed a dull olive-drab appearance.

Sample 6—This sample was placed on screens and dried by means of an electric fan in a dark room at room temperature,

which was much lower than the temperature of the sun-dried samples.

In testing these samples, the alfalfa was fed at levels ranging from 0.125 to 2 per cent. The results of these tests are given in Chart 2. In comparing the three samples, it is again seen that treating the material at a high temperature, as used with Sample 5, was not destructive to vitamin A. If comparisons had been limited to the sun-dried sample (Sample 4) and the sample dried in the dark (Sample 6), it might have appeared that the sun's rays were responsible for the marked destruction of vitamin A in Sample 4. But since Sample 5, which had also been exposed to the action of the sun for a similar period as Sample 4, was found to be superior to either of the other samples, the sun's rays (ultra-violet rays)

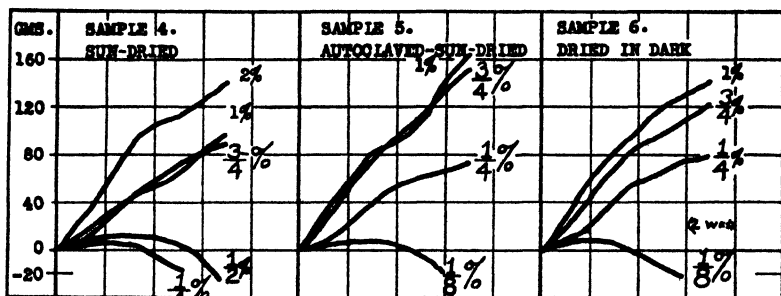


CHART 2. Composite growth curves of groups of rats showing the influence of various treatments on the vitamin A content of alfalfa.

would not seem to be the destructive factor. It therefore appears that the heating prior to exposure to the sun (Sample 5) affected some factor within the plant tissue responsible for the destruction of vitamin A, possibly enzymes which would be inactivated at sterilization temperatures. If enzymes are responsible for the vitamin A destruction, the relatively lower vitamin content of Sample 4 as compared to Sample 6 must be due to a more favorable temperature for enzyme activity during the drying period.

The higher vitamin A content of the samples of Experiment II may be due to the fact that in Experiment I the samples were from mature hay while those of Experiment II were from about half grown alfalfa.

Experiment III—The results of the second experiment indicated that enzymes may play an important rôle in vitamin A destruction in alfalfa. To test this further, it was necessary to take one sample in which the enzymes were inactivated, such as Sample 5, and another in which they would still be active, such as Sample 6, and treat them under conditions favorable to enzyme action.

Sample 5A—To a weighed portion of Sample 5, which had been autoclaved, was added an equivalent amount of water. The moistened alfalfa was placed in a muslin bag and suspended in a closed system with a moist atmosphere. A small amount of toluene was placed in a beaker beneath the sample which tended to inhibit the development of micro biological organisms but not

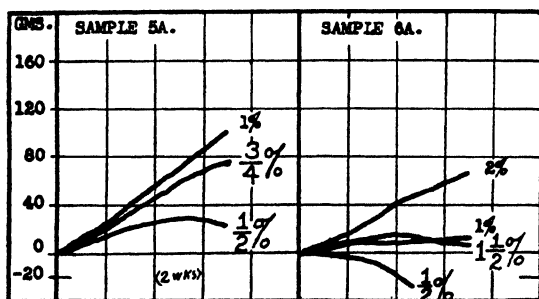


CHART 3. Composite growth curves of groups of rats showing the influence of enzyme activity on vitamin A in alfalfa. Each sample was treated under conditions optimum for enzyme activity. Sample 5A was previously heated to inactivate enzymes, while Sample 6A was not so treated.

repress enzyme activity. The sample was then incubated at 38° for 24 hours and finally dried in an oven at 60°.

Sample 6A—A portion of Sample 6, which had been dried in the dark at room temperature, was treated in the same manner as described above.

The effect of this treatment is shown in Chart 3. It is seen that Sample 6A, which had been dried in the dark with no previous heating, suffered greater destruction of vitamin A through incubation than did Sample 5A in which the enzymes had been inactivated by heat. Thus, it seems logical to conclude that enzymes play an important rôle in the destruction of vitamin A in the curing of alfalfa.

DISCUSSION

In developing processes for the artificial drying or dehydration of hay materials, it is of great importance to know whether or not such processes are destructive to the nutritive value of the products so treated. It is equally important to know what factors influence the preservation or destruction of any nutritive factor.

Past experiences have revealed that in the usual field curing methods, practices which tend to preserve the green color of hay yield the more nutritive products, and as a result for all practical purposes hays have been evaluated largely on this basis. With the use of artificial drying processes, the green color of plant materials is preserved to a greater degree than is possible by natural processes.

If the bleaching of the green color as well as the destruction of vitamin A by the field curing process is due to oxidation, the preservation of the green color in the machine-dried samples would indicate that the oxidation of these samples had been greatly reduced. Furthermore, it would indicate that Sample 3, which was rapidly dried in an atmosphere of flue gas, suffered from oxidation to a lesser degree than Sample 2. However, the biological tests indicate there is practically no difference in the vitamin A content of the two machine-dried samples although there was a difference in color intensity. It is interesting to note that, while bright green color is associated with quality in field-cured alfalfa, green color is not necessarily an index of vitamin A preservation as is evidenced by Sample 5 which lost its bright green color during the autoclaving process and became olive-drab in color without apparent destruction of the vitamin.

These experiments clearly demonstrate that the practice of treating alfalfa at elevated temperatures in mechanical dryers, is without deleterious effect on vitamin A. Even a temperature as high as the hot flue gas used in one of the machines, or the sterilization temperature of the autoclave, was not destructive. This is in agreement with Steenbock and Boutwell (9) who concluded that vitamin A as found in plants is comparatively stable to high temperatures.

The deterioration of vitamin A in the field-cured alfalfa as compared with the machine-dried samples, naturally raised the question as to the responsibility of the sun's rays (ultra-violet

rays) in the destruction of the vitamin. The fact that drying alfalfa in the dark (Sample 6) produced a sample superior to the sample dried in the sun (Sample 4), seemed to confirm this theory. But when it was found that by simply treating alfalfa in an autoclave prior to exposure to the action of the sun's rays a superior product was produced (Sample 5), the possibility of a photochemical reaction as being responsible for the destruction of vitamin A was eliminated.

The data presented in Experiments II and III indicate that enzymes may play an important rôle in the destruction of vitamin A in alfalfa. Since enzyme activity may be accelerated by proper temperature and by adequate moisture, and, also, since enzyme action may be inhibited by desiccation, by lowered temperature, and completely stopped by destroying the enzyme as by heat, these facts seemed to offer a possible explanation for the vitamin A behavior in the samples tested in these experiments. Thus in the field curing method, temperature is made favorable by the heat of the day, moisture is present in the plant tissue, and the drying is relatively slow, all contributing to vitamin A deterioration. With dew, rain, or even humid climatic conditions, there may be a repetition of these conditions, followed by further destruction of vitamin A. Under extremely dry atmospheric conditions, the drying would be rapid and possibly the vitamin destruction would not be as great. Sample 4, which was dried in the absence of dew, had only the one set of conditions to affect it. In the sample that was dried in the dark the enzyme activity was repressed by a relatively lower temperature and gave a more potent vitamin A product.

In the mechanical drying processes, the enzymes are quickly inactivated by heat and the drying is rapid. As a result, a product highly potent in vitamin A is produced, as shown by Samples 2 and 3. In Sample 5, which was sterilized before exposure to the sun, the enzymes were inactivated by high heat which prevented their action under the favorable conditions of moisture and temperature during the drying period in the sun.

In view of the inactivation of enzymes during mechanical drying with heat, it would be expected that a product so produced would retain its vitamin A value during storage. This was found to be the case when tests which were made on Sample 3 a year later

than those reported indicated that the vitamin had not suffered a measurable deterioration.

SUMMARY

1. In comparing two processes of artificial drying of alfalfa with the field curing method, it was found that artificial drying tends to preserve the vitamin A content of alfalfa while the field curing process is considerably destructive to vitamin A.

2. Mechanical drying with either heated air or hot flue gas appeared to be equally effective in preserving the vitamin A content of alfalfa.

3. Studies to determine the factors involved in the preservation or destruction of vitamin A in these processes showed that high temperatures, such as are used in mechanical drying, are not destructive to vitamin A, and also, that the sun's rays (ultra-violet rays) are not responsible for the destruction of vitamin A during the field curing process.

4. Evidence is presented which indicates that enzymes are the important factor in the destruction of vitamin A during the field curing process.

5. Conditions which favor enzyme activity, such as were encountered in the field curing process, tend to lower the vitamin A value in alfalfa, while conditions adverse to enzyme activity, as in the mechanical drying process, tend to preserve vitamin A.

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THE EFFECT OF ACID DENATURATION UPON THE COMBINING POWER OF FIBRINOGEN

BY MARION FAY AND BYRON M. HENDRIX

(From the Laboratory of Biological Chemistry, School of Medicine, University of Texas, Galveston)

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In a study of the acid- and base-binding capacity of fibrin and fibrinogen, it became evident that the latter is changed by long standing in the presence of a small amount of acid, even when the solution is kept in the refrigerator. The change is shown by the greater tendency of the protein to precipitate. Unless the protein has been previously dialyzed against sodium chloride solution, it forms an insoluble jelly-like precipitate soon after the addition of enough hydrochloric acid to bring the pH to 2.5 or 3.0. This precipitate usually does not form if the protein has been dialyzed. That the dialyzed fibrinogen is also changed by acid is shown by the appearance of a precipitate when the acid is neutralized. This precipitate does not dissolve readily in dilute alkali, whereas native fibrinogen is easily dissolved by a small amount of sodium hydroxide. The fibrinogen is so readily changed by the addition of acid that 0.1 N HCl usually precipitates some of the protein immediately in an insoluble form. A more dilute acid (0.02 N) in equivalent amount may not cause immediate precipitation, but if the fibrinogen solution has not been previously dialyzed, the precipitate is formed after standing several hours in the cold.

The process of heat denaturation of protein has been explained by Robertson (1), by Sørensen (2), by Chick and Martin (3), and others as a dehydration. This dehydration, according to Robertson, is analogous to the loss of water of crystallization by certain salts. Wu and Wu (4) thought that they had evidence to show that hydrolysis of certain peptide linkages occurred during the process of coagulation. More recently, Wu (5) has concluded that such hydrolysis is not an essential part of coagulation. P. S. Lewis (6) suggested that hydrolysis occurs during coagulation of proteins but that this hydrolysis does not involve the peptide linkage. W. C. M.

Lewis (7) had previously advanced the idea that coagulation was brought about by the opening of some bond in the protein molecule other than the —CO—NH— bond but definite evidence of the nature of such a bond is not given. Booth (8) has titrated coagulated and uncoagulated egg albumin in the presence of ammonium sulfate and found no reason to believe that the acid- and base-combining power of that protein is changed during the process of heat coagulation. Hendrix and Wilson (9) found that coagulated egg albumin combines with less acid or base than the uncoagulated protein. Booth coagulated his protein at pH 6.99 and on a boiling water bath where the temperature probably did not reach 100° . Hendrix and Wilson heated their material at the isoelectric point in an autoclave at atmospheric pressure for 30 minutes.

Denaturation of protein by acid has not received the same attention as heat denaturation. Wu and Wu (10) investigated the effect of acids and alkalis upon several proteins and found that, in general, the solubility of the protein was altered. Cubin (11) has studied the denaturation of oxy-hemoglobin and of egg albumin at $18\text{--}37^{\circ}$, at various hydrogen ion concentrations. Hemoglobin is rapidly denatured at pH 4 even at 18° , while egg albumin is only slowly denatured at pH 1 unless the temperature is about 37° . Cubin gives no evidence to show whether the acid denaturation of protein is similar in effect to heat denaturation.

The results presented in this paper are of interest in that they show that denaturation of a protein by dilute acid produces an effect upon the acid- and base-combining power similar to that of heat denaturation as carried out by Hendrix and Wilson.

Methods

Preparation of Fibrinogen—Beef blood was collected over potassium oxalate at the slaughter-house, brought to the laboratory, and stored in the ice chest. This blood was centrifuged as rapidly as possible but at least 2 days were necessary to centrifuge the entire sample. The usual difficulties in obtaining a clear plasma from beef blood were encountered, thus making necessary several precipitations of the fibrinogen. The precipitations were carried out with sodium sulfate, the final concentration being about 10 per cent, as suggested by Howe (12). 300 gm. of sodium sulfate were dissolved with the aid of heat in water, filtered, the volume adjusted to 750 cc., and the solution at a temperature of 40° poured into 3 volumes of the cold plasma. The copious precipitate was separated by centrifuging, washed several times with half saturated sodium chloride solution, and reprecipitated. Only

rarely were two precipitations sufficient, usually three being necessary to remove the hemoglobin. The final precipitate was washed well in half saturated sodium chloride, dissolved in the dilute saline, filtered, and analyzed for nitrogen (Kjeldahl) and for chloride (Volhard-Harvey). Sometimes difficulty in redissolving the protein was encountered after the third precipitation. A few cc. of 1 per cent sodium bicarbonate served to dissolve the fibrinogen in such cases. Dialysis was made part of the routine procedure in the later part of these experiments, in order to make sure that no sulfate remained in the solution, although repeated analyses had shown none to be present. Before filtration, the protein was rapidly dialyzed against running sodium chloride solution, about 18 liters being used. It was then placed in the ice chest and allowed to dialyze overnight against large amounts of the saline. The next morning the protein was again dialyzed against 18 liters of running saline, filtered, and the analyses made. From the nitrogen analysis the protein content of the solution was calculated with the factor 6.25 and volumes containing 0.5 gm. of fibrinogen were measured out.

This procedure usually took 4 days, 2 for centrifuging, 1 for precipitation and purification, and 1 for analysis. Throughout this time the protein was kept at a low temperature and from the physical properties and the speed of clotting when thrombin was added no denaturation seemed to take place. The protein stayed in solution very well unless through error the sodium chloride content was too low or the protein content too high. The yield varied from 6 to 22 gm. from 12 liters of blood.

Titration—The 0.5 gm. portions of protein were diluted and the sodium chloride content adjusted. The volume, no matter what the treatment, was always brought to 125 cc. and the sodium chloride was made the same for all members of a series. At first 0.9 per cent sodium chloride was used but later 1.25 per cent was found more satisfactory. After dilution one series was allowed to stand overnight in the ice chest without further treatment. To the remainder were added 35 cc. of 0.02 N HCl, the final concentration of acid thus being about 0.0056 N. This gave a pH which in various experiments ranged from 2.5 to 3.5. The acid-treated series was also allowed to stand overnight with the first series.

On the following day, varying amounts of 0.02 N NaOH were added to the acid-treated samples to give a series ranging from the original pH (2.5 to 3.5) to about pH 8. After mixing, the pH was determined immediately with either the hydrogen or quinhydrone electrode. We found that the two electrodes checked each other fairly well within the range used in this work. In experiments upon the undialyzed material, a precipitate always formed on standing with the acid, which made thorough mixing with the alkali difficult. With the dialyzed material, a heavy precipitate always formed near the isoelectric point, which again gave mechanical difficulties; but the agreement between the dialyzed and undialyzed material seems to show that both were mixed thoroughly.

Two methods were employed with the untreated material. At first, the volume was adjusted to 90 cc. to stand overnight and just before titration 35 cc. of the 0.02 N HCl were added, plus the appropriate amount of NaOH. Later on we found that identical results were obtained if instead of adding acid plus base, the desired amount of acid or alkali was added directly to the protein. The volume difference was adjusted by means of a control. The method of Tague (13) was used, control determinations of sodium chloride solution being made and the amount of acid or alkali necessary for the control subtracted from the protein figure. Thus the curves (Charts I and II) represent volumes of acid or base combined with 0.5 gm. of protein, with the isoelectric point¹ taken as zero.

¹ The isoelectric points of the acid-treated and of the untreated fibrinogen were determined by a modification of the method of Cole (14). Measured amounts of the protein solution were added to mixtures of acetic acid and sodium acetate and the pH of the tube containing the greatest precipitate determined by the quinhydrone electrode. Some difficulty in judging the amount of precipitate was encountered with the acid-treated protein, as there was a heavy precipitate in all tubes near the isoelectric point. However, the results in a number of determinations agreed quite well and the average of these various experiments was taken for the zero point on the curves. In the acid-treated protein, the isoelectric point shifted towards neutrality as would be expected. The values obtained were 4.686 for the untreated and 5.20 for the acid-treated. The determinations were made on almost all of the untreated preparations and on all the acid-treated samples which did not precipitate.

In some instances the untreated protein and the acid-treated protein were analyzed for amino nitrogen by Van Slyke's manometric method.

DISCUSSION

In Chart I are plotted the results of eleven titration experiments upon untreated fibrinogen. Chart II shows the titrations upon the corresponding acid-treated samples. The probable curves have been drawn in both charts. The observed variations are probably caused by varying amounts of impurities in the fibrinogen samples. The original pH of the solutions before treatment ranged from 5.67 to 6.99. The mechanical difficulties in preparing a pure fibrinogen solution are great since its chief difference from other globulins is the reaction with thrombin. In testing a solution with thrombin, globulins may be carried down with the coagulum. In certain samples the serum from a stiff clot would repeatedly show further coagulation upon additions of thrombin, thus making difficult a quantitative determination by the clotting method.

The difference in the curves in Charts I and II shows that the acid- and base-combining power of the acid-treated fibrinogen is less than that of the untreated fibrinogen. The extent of this decrease is shown readily by the use of Van Slyke's formula (15) for buffer value. When β is the buffer value, ΔB is the increase or decrease in base and ΔpH is the change in pH. $\frac{\Delta B}{\Delta pH} = \beta$. With an increase or decrease in base equivalent to 1 cc. of 0.02 N base as the unit for ΔB and a shift of 1 pH as the unit for ΔpH , β has been calculated for the untreated and for the acid-treated protein between pH 3 and 8.5. The results are given in graphic form in Chart III. The buffer value for untreated fibrinogen is higher throughout the whole range of our titrations. This shows distinctly that more base is necessary to change the pH 1 unit in an untreated fibrinogen solution than in an acid-treated solution. Since β is the slope of the curve the results are not affected by any error in the determination of the isoelectric point of the protein.

The difference in the buffer value of the acid-treated and the untreated fibrinogen appears to be greatest in the region of pH 3 and least from pH 8 to 9. These are the regions where the com-

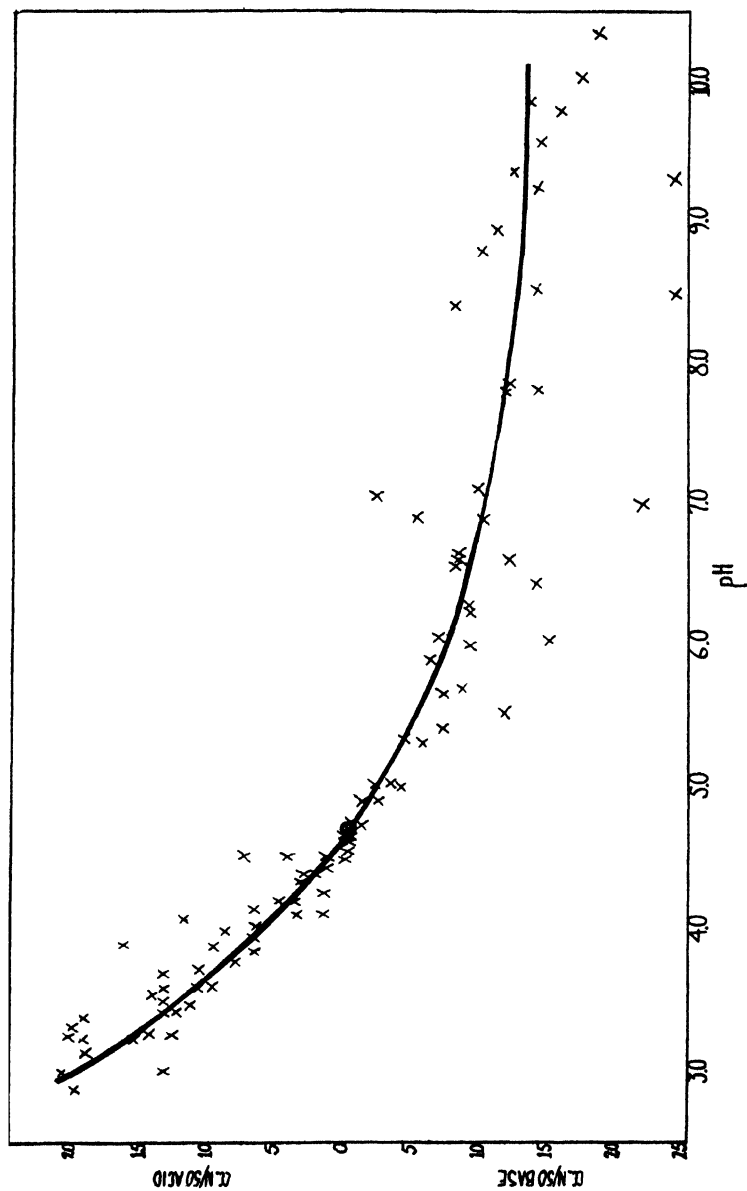


CHART I. Composite of eleven curves, showing the volume of 0.02 N HCl or NaOH combined with 0.5 gm. of untreated fibrinogen, with isoelectric point at pH 4.686.

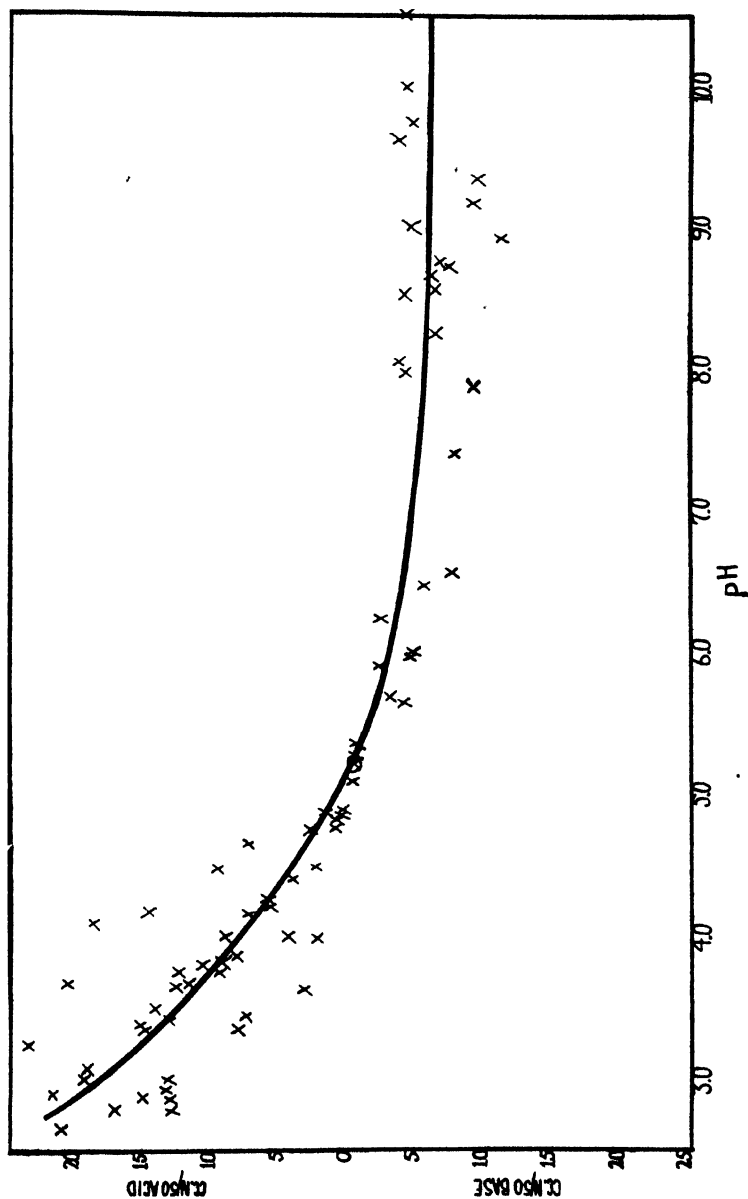


CHART II. Composite of eleven curves, showing the volume of 0.02 N HCl or NaOH combined with 0.5 gm. of acid-treated fibrinogen, with isoelectric point at pH 5.20.

posite curve is least accurate. In the high acid range, the slope of the curve towards zero found in the individual combination curves tends to be lost in the composite curve. On the alkaline side, the titration enters the region where the quinhydrone electrode is less accurate. Therefore β can be most accurately compared in the region from pH 4 to 7.5. At pH 4 the buffer value of the untreated protein is greater than that of the acid-treated by 2.86 and at pH 6.8 the difference is 1.2, showing some tendency for the values to

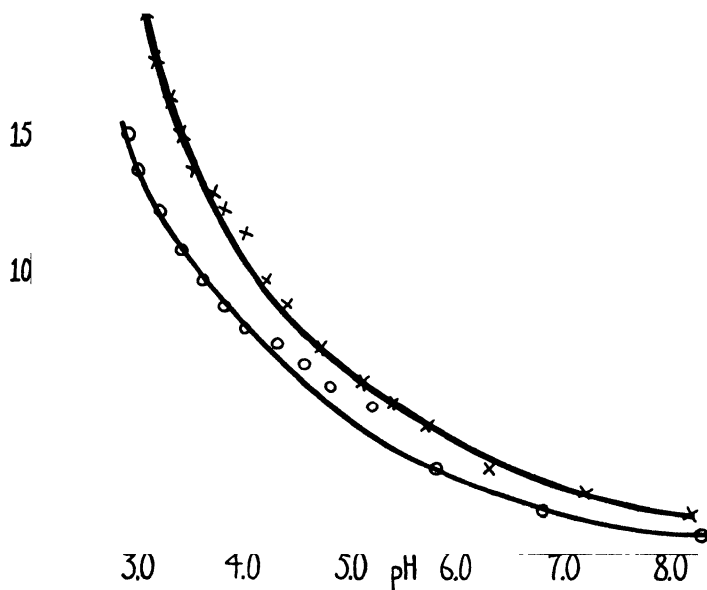


CHART III. Buffer values. \times = untreated fibrinogen; \circ = acid-treated fibrinogen.

approach each other on the alkaline side. At pH 3 the difference is nearly 7, while at pH 8.2 it is about 0.6.

The difference in acid- and base-combining power of native and of acid-denatured fibrinogen is similar to the difference in the combining power of untreated and heat-coagulated egg albumin, observed by Hendrix and Wilson. It is impossible to compare accurately the observed effect of acid treatment with that produced by heat coagulation because different proteins have been used and because the amount of the two taken for titration was

not the same. We can conclude that fibrinogen denatured by dilute acid at a low temperature shows a decrease in acid- and base-combining power similar to that observed for egg albumin coagulated at the isoelectric point by autoclaving at atmospheric pressure for 30 minutes. These results are in accord with the theory that during denaturation protein undergoes polymerization. Some confirmatory evidence for this point has been observed by determining the primary amino nitrogen of the untreated and the acid-treated fibrinogen. These determinations have not been completed but have shown about 8 per cent less amino nitrogen in the acid-treated than in the untreated fibrinogen.

SUMMARY

Fibrinogen from beef blood is denatured by standing with hydrochloric acid at pH 2.5 to 3.5 for 12 to 15 hours at 12–14°. The acid- and base-combining power of fibrinogen is diminished by this acid denaturation. The acid-denatured fibrinogen has an isoelectric point of pH 5.2, the undenatured, one of 4.7. The buffer values of the untreated and of the acid-treated protein have been calculated from the combining curves. The buffer value of the acid-treated fibrinogen is less than that of the untreated protein throughout the entire range of our titrations.

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STUDIES ON THE EFFECT OF ALKALI ON PROTEIN

I. THE OPTICAL BEHAVIOR OF "RACEMIC PROTEIN"

BY FRANK A. CSONKA AND MILLARD J. HORN

*(From the Protein and Nutrition Division, Bureau of Chemistry and Soils,
United States Department of Agriculture, Washington)*

(Received for publication, August 13, 1931)

Preliminary to a study of the optical rotation of glutelins (1), we carried out experiments on the effect of the concentration of alkali at different temperatures on protein. The chemical changes occurring in the alkali digest were followed optically and also by determining the nitrogen not precipitable with tungstic acid. The change in optical rotation is practically parallel with the decrease of protein nitrogen precipitated, pointing to a rapid disintegration of the protein molecule. These results led us to separate and study the protein cleavage products, which were called by Dakin "racemized proteins."

The polariscopic readings of these alkali digests never reached zero activity. We believed that this optical activity could be explained by the presence of optically active substances in the filtrate. Several papers have appeared in the past, by Kossel and Weiss (2), Dakin (3), and Underhill and Hendrix (4), on the effect of alkali on proteins and the chemical and physiological behavior of the products obtained. All these authors must have considered the products optically inactive or closely so, but to our knowledge their specific rotation was never determined. One may obtain from the literature a misleading conception in regard to the nature of these so called racemized proteins. For instance, the expression racemic casein naturally conveys the idea of an optically inactive protein. As a matter of fact, so called racemic casein is not casein but a decomposition product of casein, and it is optically active. This would be true even if casein is a mixture of several proteins, according to the present belief. It would

be more satisfactory at present to consider these products as optically active decomposition products of proteins and drop the word "racemized" entirely. We are not ready to suggest a specific name for them, but on account of their characteristic behavior toward acid hydrolysis as contrasted with that of native proteins they should be in a class by themselves. When hydrolyzed by acid the native proteins yield optically active amino acids, and a very small quantity of racemized amino acids. On the other hand, Dakin showed that when the protein products obtained by alkali treatment are hydrolyzed by acid, they yield racemic amino acids and only a small quantity of active amino acids.

We prefer to make a distinction between the effect of alkali on proteins and the result of acid hydrolysis of the cleavage products separated from the alkali digest. Racemization occurs in the latter procedure; the alkali produces only some unknown change in the molecule of the protein cleavage products, for which we can offer only a plausible theory, which, however, we believe is more in harmony with our experimental findings than is Dakin's. That the optical rotation of the alkali digest is less than that of the native protein could be explained satisfactorily without considering the phenomenon of racemization. The optical rotation of the alkali digest represents the sum of the optical activities of the cleavage products, which is lower than the activity of the native protein. The fact that we were unable to separate any racemic product from the alkali protein digest supports our hypothesis.

Dakin's theory of protein racemization involves the change of an asymmetric carbon atom into a symmetric carbon atom. However, the different protein fractions separated from alkali digests are all optically active. It is true that he found optically active amino acids in the acid hydrolysate of the so called racemized proteins, which he attributed to the position of some amino acid having a free carboxyl group in the protein molecule. It would be rather far fetched to assume that the optical activity possessed by these alkali cleavage products is caused by the amino acids having a free carboxyl group and that after acid hydrolysis the optical activity is practically lost instead of remaining almost constant. We say almost constant, as we realize that the part of the protein molecule to which the side chain carboxyl groups are attached undoubtedly influences the optical rotation in some meas-

ure, even though the attached groups themselves are inactive; the transitory enol formation, according to Dakin, makes them inactive.

Our explanation for the change of optical activity when protein is treated with alkali is that the protein products have a specific rotation of their own, which is different from that of the original protein. All these products obtained from the alkali digests were found to be active. We maintain that in them the asymmetric carbon atoms remain asymmetric, hence their optical activity. There must exist, however, chemically or physically a difference in the molecule of these products and in that of the native proteins, as acid hydrolysis results in optically inactive compounds in the first case and optically active compounds in the second case. This difference is probably caused by the manner in which alkali breaks down the protein molecule or shifts certain groups within it with reference to the asymmetric carbon atoms.

Levene and Bass (5) digested proteins with alkali for different time intervals. The products of these digestions were then hydrolyzed with acid. From the results obtained they concluded that racemization of proteins increased with the time they were subjected to the action of alkali. We have shown that there is a rapid disintegration of the protein molecule on treatment with alkali and interpret their findings as confirmatory evidence of our hypothesis that hydrolysis of the protein by the alkali is also a factor in the racemization phenomenon.

When a protein molecule splits, the number of side chain carboxyl groups necessarily increases if the break occurs at the CO—NH peptide linkage; therefore, if Dakin's explanation that the active amino acids found in the hydrolysate of the alkali digest represent the end carboxyl groups is correct, the splitting must occur at some other points. Considering Levene and Bass' observation mentioned above and ours, however, we may assume with Dakin that enolization of the asymmetric carbon atom occurs only on a cleavage of the protein molecule, rendering the secondary end carboxyl groups inactive. This would leave open the explanation for the presence of inactive amino acids in the hydrolysate of optically active alkali cleavage products. Our hypothesis would explain this phenomenon.

EXPERIMENTAL

Preliminary experiments had convinced us that when treated with dilute sodium hydroxide in concentrations of 0.05 and 0.5M even at room temperature (21°) native protein disintegrates and that when the temperature is raised to 38° this hydrolytic process increases very rapidly. As shown in Figs. 1 and 2, in which are given the percentages of non-protein nitrogen plotted against time, within 48 hours practically one-half of the original protein nitrogen is converted into a form not precipitable by tungstic

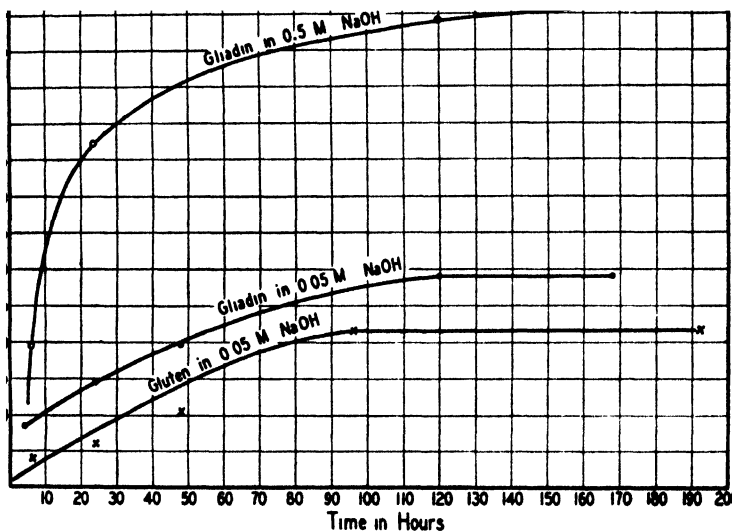


FIG. 1. Decomposition of gliadin and gluten in sodium hydroxide solution at room temperature measured by the percentage of non-protein nitrogen.

acid. It is interesting to compare these curves with the racemization curves we published in a previous communication (1) in which we stated that decrease in optical rotation may have nothing to do with racemization. Cleavage of the protein molecule by the alkali and the decrease in optical rotation represent a similar type of reaction and, as we stated above, racemization does not play any part at this stage.

3 to 5 gm. of the protein under investigation were dissolved in

0.05 or 0.5 M sodium hydroxide solution. The solution was filtered in order to remove undissolved particles, and it was then brought by additional sodium hydroxide solution to such a volume that the protein concentration was approximately 1 per cent. Aliquot parts were removed for total nitrogen determinations at the beginning of the experiment. Later at regular time intervals 10 cc. samples were removed for the non-protein determinations

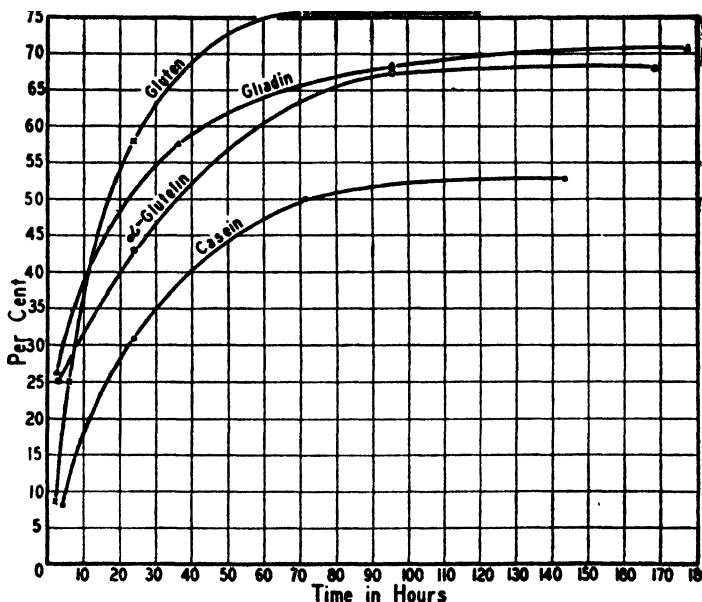


FIG. 2. Decomposition of gluten, gliadin, α -glutelin, and casein in 0.5 M sodium hydroxide at 38°, measured by the percentage of non-protein nitrogen.

as given below. After neutralization with dilute sulfuric acid, equal volumes of 10 per cent solution of sodium tungstate and 0.6 N sulfuric acid were added to precipitate the proteins as recommended for blood analysis by Folin and Wu (6). After standing for an hour, the precipitate was removed by filtration, and the nitrogen content of the filtrate was determined in duplicate according to Pregl's micro method as modified by Clark and Collip (7).

In Table I are presented data on the cleavage products separated from alkali digests of proteins. We used wheat gluten, casein, and egg albumin. As the technique varied in each case, a description of each experiment is given. We do not claim that these preparations represent individual proteins. The main purpose was to determine the optical behavior of products which, judging from the literature, we believed to be optically inactive. We were more interested in the nature of the alkali effect on the proteins than in the yields of each preparation. All fractions showed the characteristic biuret color reaction.

TABLE I

Properties of Protein Cleavage Products Obtained by Treating Protein with 0.5 M Sodium Hydroxide at 38°

Protein used and time digested	Preparation No.	N on moisture-free and ash-free basis	Specific rotation	
			In 0.5 M NaOH	After hydrolysis with 20 per cent HCl
		<i>per cent</i>	<i>degrees</i>	<i>degrees</i>
Gluten digested for 52 days	1	14.26	-67.5	+8.6
	2	12.45	-98.2	0
	3	13.83	-97.9	+2.7
	4	14.64	-76.9	0
	5	13.47	-69.2	0
Casein digested for 36 days	1	12.40	-45.8	0
	2	15.20	-30.9	0
	3	13.40	-46.0	0
Egg albumin digested for 21 days	1	13.20	-25.8	+2.9
	2	15.00	-19.8	+2.2

Gluten

Preparation 1—This was obtained by slight acidification of the alkali digest, which had stood for 52 days at 38° (Filtrate A). The precipitate was redissolved in 0.5 M sodium hydroxide, and the clear solution was diluted with distilled water to bring the alkali concentration to approximately 0.05 M. Solid ammonium sulfate was then added to make the solution 35 per cent saturated. The precipitate thus obtained was separated by centrifugation.

(Filtrate X), redissolved by the addition of water (sufficient alkali being present), and reprecipitated by slight acidification. The precipitate was washed free from salt by distilled water and was dried *in vacuo* over sulfuric acid.

Preparation 2—This could be obtained either by saturating Filtrate X by the addition of solid ammonium sulfate, or by acidification. The latter procedure was used. The precipitate dissolved almost completely in 70 per cent ethyl alcohol. The solution was filtered clear and evaporated *in vacuo* to a sirupy consistency. When treated with ether, the product was converted to a light yellowish powder.

Preparation 3—To Filtrate A alcohol was added until 80 per cent alcohol concentration was reached. The precipitate, which consisted mostly of inorganic salts, was washed with 80 per cent alcohol, the washings being added to the filtrate. The alcohol was removed by distillation *in vacuo*. After acidifying, the resulting precipitate was washed with 70 per cent alcohol and finally dehydrated by treatment with absolute alcohol, followed by treatment with ether.

Preparation 4—The filtrate from Preparation 3 was freed from sulfuric acid by adding an equivalent amount of barium hydroxide. The liquid was concentrated *in vacuo* to a small volume, and enough alcohol was added to bring the alcohol concentration up to 86 per cent. After it had stood in a refrigerator for several weeks, a brown sirup separated. The supernatant liquid was decanted, and the sirup was dehydrated in the usual manner with alcohol and ether.

Preparation 5—The supernatant liquid from Preparation 4 was evaporated *in vacuo* to a sirup and dehydrated with absolute alcohol and ether.

Casein

The protein in approximately 1 per cent concentration was digested in 0.5 M sodium hydroxide for 36 days at 38°, after which it was acidified with dilute sulfuric acid. The precipitate was washed and dehydrated (Preparation 1). The liquid was brought to 70 per cent alcohol concentration, and most of the inorganic salts were removed by filtration. The filtrate was evaporated *in vacuo* to a small volume, and 6 volumes of absolute alcohol were

added. On standing, a sirup separated. This was dried *in vacuo* over concentrated sulfuric acid (Preparation 2). The supernatant liquid which was decanted from the sirup was evaporated to dryness. The residue is Preparation 3.

Egg Albumin

The procedure was similar to that used for casein in Preparations 1 and 2.

SUMMARY

Wheat gluten, casein, and egg albumin were digested with 0.5 M sodium hydroxide at 38°. The products which were separated from the alkali digest were optically active, but when boiled with 20 per cent hydrochloric acid they yielded hydrolysates which were practically optically inactive. It is suggested that the name "racemized protein" for these alkali digestion products be discontinued because it is misleading. Dakin's theory of keto-enol formation is discussed, and the conclusion is reached that it does not give a satisfactory explanation for the observations reported in this paper.

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A NEW COLORIMETRIC METHOD FOR THE ESTIMATION OF POTASSIUM

By H. R. D. JACOBS* AND WILLIAM S. HOFFMAN†

(From the Department of Medicine, Rush Medical College, the University of Chicago, Chicago)

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The usual method for the estimation of potassium in the blood and the urine depends, as is well known, upon the precipitation of the element as the cobalti-nitrite and the subsequent estimation of one of the constituents of the precipitate. The reducing power of the precipitate is used by Kramer and Tisdall (1); the formation of a diazo color by the nitrite is employed by Doisy and Bell (2); the potassium is titrated as the acid tartrate by Fiske and Litarczek (3); the cobalt is determined as the thiocyanate by Breh and Gaebler (4).

From a theoretical point of view those methods which determine directly a stable constituent of the precipitate are preferable to those which depend upon the activity of the unstable nitrite radical. The methods of Fiske and Litarczek and of Breh and Gaebler embody this principle, but each of these has a disadvantage: that of Fiske and Litarczek involves an ashing and a second precipitation, and that of Breh and Gaebler depends upon a foreign factor (alcohol concentration) as well as upon the factor being estimated.

It is the object of this paper to describe the formation of a new stable, colored compound of cobalt and its application to the micro estimation of potassium in biological fluids.

Method

Principle—When a solution of sodium ferrocyanide is added to a mixture of a cobaltous salt and choline hydrochloride in water

* Ernest E. Irons Fellow in Medicine; under the supervision of Dr. George F. Dick.

† National Research Council Fellow in Medicine; under the supervision of Dr. Wilber E. Post.

solution, an emerald-green color develops immediately. Preliminary experiments with this color showed that it was sensitive to minute amounts of cobalt, choline, and ferrocyanide, and that, in the presence of a given excess of choline and ferrocyanide, it could be used to determine colorimetrically small variations in the concentration of cobalt. It was further established that the colored solution might be diluted with water without affecting its quality, and that it developed to maximum intensity in a few minutes and maintained this intensity for hours.

Procedure—The precipitation of potassium from blood serum is carried out according to the method of Kramer and Tisdall. Into a 15 cc. conical centrifuge tube marked at 6 cc. is pipetted 1 cc. of serum. 2 cc. of Kramer and Tisdall's sodium cobalti-nitrite reagent¹ are added slowly with constant agitation of the serum. After 45 minutes, 2 cc. of water are added, the contents mixed, and the tube centrifuged at moderate speed for 15 minutes.

The tube is then inverted and drained briefly. 1 cc. of water is run down the side of the tube, the tube centrifuged 5 minutes, and again inverted and drained. The precipitate is then suspended in 2 cc. of 70 per cent alcohol with the aid of a stirring rod, the tube centrifuged 5 minutes, inverted, and drained. The alcohol washing is repeated once.

After the tube has drained for a few minutes, the precipitate is suspended in 1 cc. of water with the aid of a stirring rod. Another cc. of water is added and the tube then immersed in a boiling water bath for 10 minutes. The contents of the tube are agitated several times during the heating in the bath. The precipitate will dissolve to form a clear, colorless solution. A small amount of insoluble material, probably protein, does not interfere. When the tube has cooled, 1 cc. of 1 per cent choline hydrochloride and 1 cc. of 2 per cent sodium ferrocyanide are added to the solution and made up to the 6 cc. mark.

¹ *Sodium Cobalti-Nitrite Reagent*—Solution A: 25 gm. of cobaltous nitrate crystals are dissolved in 50 cc. of water and to this solution are added 12.5 cc. of glacial acetic acid. Solution B: 120 gm. of sodium nitrite (potassium-free) are dissolved in 180 cc. of water. This gives a total volume of about 220 cc. To all of Solution A are added 210 cc. of Solution B. An evolution of nitric oxide gas occurs at once. Air is drawn through the solution until all the gas has passed off. The reagent (thus prepared it has a pH of 5.7) is placed in the ice chest and filtered each time before using. It will keep at least 1 month.

The precipitation of potassium from ashed or inorganic solutions is made from 2 cc. of the unknown solution with 1 cc. of the reagent. The first washing with water after centrifugation is omitted, and the precipitate washed twice with 70 per cent alcohol as outlined for serum. The remainder of the procedure is the same.

The colored solution thus formed is compared with a standard colored solution made from 1 cc. of standard cobalt nitrate (or sulfate), 1 cc. of 1 per cent choline hydrochloride, and 1 cc. of 2 per cent sodium ferrocyanide made up to 6 cc. with water. The standard cobalt solution is made from either the nitrate or the sulfate: 0.6701 gm. of $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ or 0.6469 gm. of $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ made up to 1000 cc. with water. These solutions must be standardized because these cobalt salts are hygroscopic. The standardization is made against the cobalt contained in the precipitates of potassium from a standard potassium sulfate solution containing 0.4011 gm. of K_2SO_4 per liter (equivalent to 18 mg. of K per 100 cc.).

Comment

This method embodies the theoretical advantage of determining directly a stable constituent of the potassium precipitate. The procedure is relatively simple. The use of a permanent standard permits of more accurate comparisons between values obtained at different times, and serves as a check upon the efficiency of the precipitating procedure when this is in question.

There are only two accurate measurements to be made in each determination: that of measuring 1 cc. of the serum to be used and that of diluting the colored solution to the 6 cc. mark. All of the manipulations are carried out in the original tube so that there is no loss from transfers.

Washing the precipitate in alcohol has the advantage that it is thoroughly freed from the precipitating agent without danger of loss, since it is insoluble in alcohol. The alcohol also makes the precipitate cohere well so that the supernatant fluid may be poured off and the tube drained upside down. It was found that there was a large amount of cobalt in the first water washing, a small amount in the first alcohol washing, a trace in the second alcohol washing, and none in the combined third alcohol washings

from eight tubes in a test experiment. When the precipitates are washed with water according to the method of Kramer and Tisdall, even the fourth washings contain perceptible amounts of cobalt, which must come either from the residual precipitating reagent or from solution of the precipitate itself.

The quantity of cobaltous salt to be used in the preparation of the standard cobalt solution is calculated from the formula of the precipitate, $K_2NaCo(NO_2)_6 \cdot H_2O$, in which the amount of potassium is to be 0.18 mg. (equivalent to that in 1 cc. of solution containing 18 mg. per 100 cc.). Cobaltous nitrate and sulfate rapidly absorb water during exposure to air, so that weighing is not accurate, and standardization against a known potassium solution is necessary. Once made, the cobalt solution keeps indefinitely. If the sulfate is used for the standard cobalt solution, it may also be standardized by determining the sulfate in the solution.

The choline solution is made from choline hydrochloride crystals. A stock solution of 10 per cent, from which the 1 per cent solution is made by dilution, may be kept in the refrigerator. Dry crystals may be conveniently prepared by precipitating an alcoholic solution with ether.

The sodium ferrocyanide is made in 2 per cent solution from $Na_4Fe(CN)_6 \cdot 10H_2O$. It is preserved in a cool, dark place. When it becomes very yellow or when it develops a red precipitate, it should be discarded. Of course, potassium ferrocyanide may be used instead.

The quantities of choline and ferrocyanide used in this method are sufficient for any amounts of cobalt encountered in potassium determinations. In developing the color, the choline solution should be added to the cobalt solution first, and then the ferrocyanide. If the ferrocyanide is added to the cobalt solution before the choline, the color develops much more slowly, instead of immediately, as in the given procedure.

The object in adding 1 cc. of water as the first washing after the centrifugation is to remove as much of the protein-containing fluid as possible before washing the precipitate with alcohol. The presence of too much coagulated protein makes disintegration of the precipitate somewhat more difficult.

If the precipitate is dissolved as outlined above, solution will be rapid and complete. The small particles which sometimes remain

are probably protein, but they give up their salt readily and become translucent flakes which cause no difficulty. They settle

TABLE I
Determinations of Potassium in Known Potassium Solutions

K taken	K found		K taken	K found
	I	II		
<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
0.10	0.1043	0.0991	0.16	0.1666
0.10	0.1064	0.0999	0.16	0.1600
0.11	0.1100	0.1098	0.18	0.1786
0.11	0.11*	0.1091	0.18	0.1830
0.12	0.1204	0.12*	0.20	0.20*
0.12	0.1204	0.1207	0.20	0.2012
0.14	0.1433	0.1476	0.24	0.2420
0.14	0.1386	0.1363	0.24	0.2400

* The solution against which the other solutions of the series were compared.

TABLE II
*Determinations of Potassium in Human Blood Sera**

Case No.	Potassium			Case No.	Potassium		
	Authors' method	Kramer and Tisdall method	Authors' method after ashing		Authors' method	Kramer and Tisdall method	Authors' method after ashing
	<i>mg per cent</i>	<i>mg per cent</i>	<i>mg. per cent</i>		<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>
1	19.9	17.0	19.7	6	18.6	16.6	17.1
	19.9	18.5	19.9		18.7		
2	18.4	19.6		7	19.4	19.4	
		18.2			19.7	19.0	
3	21.1			8	21.9	19.9	
	21.3				21.9		
4	21.4	19.9		9	22.3	20.2	21.7
	21.4				22.1	20.7	
5	21.3	20.2		10	20.6	19.8	20.0
	21.3				20.9		

* Ten consecutive cases.

out after the colored solution is formed and remain in the tube when the solution is poured into the colorimeter cup.

Color comparisons are best made by daylight. The colors are easily matched in any good colorimeter.

Tables I to IV present the results of determinations carried out by this method upon several types of solutions. Tables III and IV show that known amounts of potassium added to urine and serum can be determined accurately.

TABLE III
Determinations of Potassium in Urine with Added Potassium

K in urine	K added	Total K	K found
<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>
178.1	24.0	202.1	204.1
178.1	48.0	226.1	226.2
178.1	72.0	250.1	257.6
178.1	96.0	274.1	277.4

The known amounts of potassium were added to measured portions of the urine, the mixtures then ashed, and the potassium determined in an aliquot of the solutions of the ashes. Two cobalt standards, one equivalent to 18 mg. of K per 100 cc., and the other to 36 mg. of K per 100 cc., were used.

TABLE IV
Determinations of Potassium in Serum with Added Potassium

Solution A				Solution B			
K in serum	K added	Total K	K found	K in serum	K added	Total K	K found
<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>
18.6	1.8	20.4	19.9	22.2	1.8	24.0	24.0
18.6	3.6	22.2	22.2	22.2	1.8	24.0	23.8
18.6	5.4	24.0	24.1	22.2	3.6	25.8	25.9
18.6	7.2	25.8	25.9	22.2	5.4	27.6	27.5
				22.2	7.2	29.4	29.8

Ammonium salts interfere in this determination because ammonia also forms a very insoluble cobalti-nitrite precipitate. For this reason glassware should be cleaned shortly before the precipitation is made, and, for this reason also, fluids, such as urine, which contains appreciable amounts of ammonia, must first be ashed.

In serum potassium determinations it must be remembered that

the red corpuscles contain relatively much more potassium than the serum, and that potassium values will be incorrect in hemoglobin-tinged serum and in serum which has been in contact with the clot for a long time. It is best to collect the blood in perfectly dry (preferably baked) glassware, allow it to clot in an incubator for 15 minutes, and then centrifuge it, and separate the serum at once.

SUMMARY

1. The formation of a hitherto unmentioned colored solution from cobalt, choline, and ferrocyanide is described.

2. A method is outlined for the determination of potassium in blood, urine, and ash solutions by means of this color.

3. Data on determinations of potassium in blood sera, in urine, and in inorganic solution are presented.

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THE FATE OF THE ANTIRACHITIC FACTOR IN THE CHICKEN

I. THE ANTIRACHITIC FACTOR BALANCE IN THE GROWING CHICK* †‡

BY DANIEL KLEIN§ AND WALTER C. RUSSELL

(From the Department of Agricultural Biochemistry, New Jersey Agricultural Experiment Station, New Brunswick)

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Irradiated ergosterol is not as effective as cod liver oil for the prevention of leg weakness in the growing chick when fed in amounts equivalent to the latter in antirachitic potency or even several times this strength as standardized by the rat assay method (1-3). An attempt to elucidate this behavior led to a study of the fate of these two sources of vitamin D in the body of the chick.

The object of the present investigation was to ascertain how much of the antirachitic factor from each source was stored in the body of the chick, and how much was eliminated in the droppings.

Experimental Methods

Feeding of Chicks and Collection of Droppings—Eight White Leghorn chicks were put on the feeding experiment 48 hours after hatching. They were weighed individually, divided into two groups of four each, and placed in specially constructed cages (Fig. 1). These were built in such a manner that the food which the chicks scattered could be recovered, and that all of their droppings would fall upon a glass plate.

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‡ Presented before the Division of Biological Chemistry at the Buffalo meeting of the American Chemical Society, September, 1931.

§ Acetol Products Industrial Fellow. This investigation was conducted under a fellowship grant from the Newport Chemical Company, Milwaukee.

The basal ration employed had the following composition: 47 per cent ground yellow corn, 20 per cent wheat middlings, 15 per cent wheat bran, 5 per cent dried buttermilk, 5 per cent meat scrap (55 per cent protein), 4 per cent fish meal, 3 per cent ground oyster shell, and 1 per cent NaCl. The percentage of calcium in the ration was 1.80 per cent, and of phosphorus 0.87 per cent; the ratio of Ca:P being 2.

Group 1 received 1 cc. of corn oil solution containing 75 rat units of irradiated ergosterol to every 100 gm. of food, and Group 2 was supplied with 1 cc. of cod liver oil or 23 rat units to every 100 gm. of ration. The two antirachitic substances were carefully assayed biologically at the time this experiment was in progress. The supplemented chick rations were made up each week and the food remaining in the feeding troughs on the 8th day was discarded.

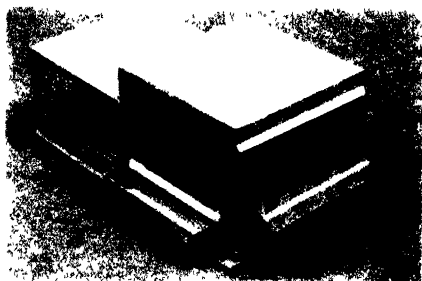


FIG. 1. Cage used for balance studies with the chick

A record of the daily food consumption, Table I, was kept for the 27 days of feeding. Any of the ration which was scattered could fall only on the glass plates. These few gm. were brushed back twice in 24 hours into the feeding troughs.

The chicken droppings were carefully removed from the glass plates twice in 24 hours by means of two steel spatulas and preserved under ethylene dichloride in brown bottles. They were stored in a refrigerator for not longer than a 2 weeks period before saponification and concentration. The urine, which formed white caps on the feces, could not be separated from the latter, but it formed only a very small fraction of the collected droppings. The latter remained on the glass during intervals varying from 3 to 20 hours.

TABLE I
Consumption of Food and Antirachitic Factor

Age	Group 1, irradiated ergosterol			Group 2, cod liver oil		
	Total daily food intake of 4 chicks	Average daily food intake per chick	Average daily anti-rachitic factor intake per chick	Total daily food intake of 4 chicks	Average daily food intake per chick	Average daily anti-rachitic factor intake per chick
days	gm.	gm.	rat units	gm.	gm.	rat units
3	9	2.2	1.6	13	3.2	0.7
4	19	4.7	3.5	16	4.0	0.9
5	27	6.8	5.1	21	5.3	1.2
6	38	9.5	7.1	34	8.5	2.0
7	32	8.0	6.0	29	7.2	1.7
8	39	9.7	7.3	31	7.8	1.8
9	62	15.5	11.6	51	12.7	2.9
10	34	8.5	6.4	30	7.5	1.7
11	52	13.0	9.7	49	12.3	2.8
12	46	11.5	8.6	50	12.5	2.9
13	35	8.8	6.6	38	9.5	2.2
14	68	17.0	12.7	67	16.7	3.8
15	69	17.2	12.9	73	18.3	4.2
16	59	14.8	11.1	28	7.0	1.6
17	84	21.0	15.7	91	22.7	5.2
18	73	18.2	13.6	78	19.5	4.5
19	77	19.3	14.5	80	20.0	4.6
20	73	18.2	13.6	70	17.5	4.0
21	96	24.0	18.0	102	25.5	5.9
22	64	16.0	12.0	52	13.0	3.0
23	85	21.3	16.0	92	23.0	5.3
24	82	20.5	15.4	74	18.5	4.3
25	97	24.2	18.1	79	19.8	4.6
26	125	31.3	23.5	90	22.5	5.2
27	129	32.2	24.1	82	20.5	4.7
28	51	12.7	9.5	55	13.7	3.2
29	108	27.0	20.2	58	14.5	3.3
Total	1733	433	325	1533	383	88
Correction for gizzard contents	-20	-5	-4	-20	-5	-1
Total	1713	428	321	1513	378	87

The chicks were to have been fed for 28 days, but one of the cod liver oil-fed birds was found dead on the morning of the 27th day. The feeding was therefore discontinued at this time. Each chick was chloroformed, then weighed, and the digestive tract down to the gizzard opened. The body weights are recorded in Table II. In none of the eight birds was food found above the gizzard. The food removed from the latter organ was considered as not eaten and that amount was subtracted from the total food consumption. The contents of the intestinal tract were allowed to remain with the carcass; the contents of the cloaca were added to the droppings.

TABLE II
Growth of Birds

	Group 1, irradiated ergosterol	Group 2, cod liver oil
	gm.	gm.
Average weight at 2 days of age	41.5	41.5
" " " 29 " " "	225	214*

* The bird found dead on the 29th day of age weighed 98 gm., and its weight was not included in the average for the group.

Saponification and Extraction—The general procedure for the extraction of the antirachitic factor was essentially the same for all materials studied. A modification of the Marcus (4) cold saponification method was employed. The saponification solution was composed of concentrated aqueous KOH in the presence of a small amount of alcohol. In the extraction of the saponified material with ethylene dichloride difficulties were encountered because of the formation of an emulsion. At first this hindrance was overcome by the use of the centrifuge, but the latter was abandoned as soon as it was noted that dilution with large quantities of tap water was a more effective way of causing the ethylene dichloride to separate. The solvent was removed under diminished pressure, the temperature of the water bath being kept between 50–70°. The non-saponifiable residues were taken up with known amounts of corn oil and the oil solutions were stored in a refrigerator, in the dark, until assayed.

Extraction of Newly Hatched Chicks—55 unfed White Leghorn

chicks were killed 48 hours after hatching, frozen at -20° for 48 hours, and then put through a meat chopper. On account of the large mass of material, only one-fifth of the saponified material was extracted, as described above, and dissolved in 22 cc. of corn oil. The results of the assay of this oil solution appear in Table III. These chicks and those used in the balance experiment were from the same lot.

Extraction of 4 Weeks Old Chicks—The carcasses of the eight chicks were stored in a refrigerator at -20° for 7 days. The frozen bodies were ground in a meat chopper and then saponified. The unsaponifiable residue from each group was taken up with 20 cc. of corn oil. The results of this assay are also displayed in Table III.

TABLE III
Assay of Antirachitic Factor in Bodies of Chicks

	Amount of corn oil solution fed	No. of chicks represented by supplement	No. of rats used	Line test
	cc.			
48 hours old chicks,	11	5½	1	++
unfed	11	5½	1	++
4 weeks old chicks				
Group 1, irradi-	5	1	2	—
ated ergosterol	10	2	1	—
Group 2, cod liver	5	1	2	—
oil	10	2	1* (rejected)	±

* This rat was rejected because it did not eat all its supplement in the 10 day period and lost 10 gm. in weight.

Extraction of Droppings—The ethylene dichloride was first removed from the droppings by filtration and the residue subjected to saponification. This procedure was followed except in the case of the droppings collected during the 1st week from the ergosterol group, which were saponified without filtration. The material remaining after the removal of ethylene dichloride from the extract of the droppings and the unsaponifiable residue from the extracted droppings of Group 1 were dissolved in 170 cc. of corn oil, and those of Group 2 in 180 cc. Table IV contains a record of the biological assay of these two oil solutions.

Vitamin D Assay—The vitamin D assay was carried out essentially as outlined by the Wisconsin Alumni Research Foundation. White rats born and reared on a modified Sherman Diet 13 (5) were used. It consisted of 60 per cent ground whole wheat, 30 per cent whole dry milk (Klim), 10 per cent Swift's meat scrap (55 per cent protein), and sodium chloride to the extent of 2 per cent of the weight of the wheat. The litters were reduced to seven at not later than 3 days of age and the rats were placed on

TABLE IV
Assay of Antirachitic Factor in Chicken Droppings

	Levels of corn oil solution fed	Per cent of total droppings represented by supplement	No. of rats used	Line test scorings; No. of rats in each group				
				=	+	++	+++	++++
	cc.							
Group 1, irradiated ergosterol	0.2	0.12	2	2				
	0.3	0.18	1	1				
	0.4	0.23	8	4	4			
	0.5	0.29	8	2	5	1		
	0.6	0.35	8		1	7		
	1.0	0.59	2			1	1	
	5.0	2.94	2					2
Group 2, cod liver oil	1.0	0.55	5	4	1			
	1.2	0.67	8	3	4	1		
	1.4	0.78	8	1	4	2	1	
	1.6	0.89	8			5	3	
	1.8	1.00	8		2	5	1	
	2.0	1.11	2		1	1		
	3.0	1.67	2				2	
	5.0	2.78	2				2	

the Steenbock rachitogenic Ration 2965 (6) when they weighed approximately 60 gm. at an age of 25 to 30 days. After 23 days on this ration, the rachitic rats were segregated in individual cages and fed 50 gm. of Ration 2965 into which the desired amount of antirachitic supplement had been thoroughly incorporated by means of an ether or oil solution. The animals were divided among the different levels of supplement according to litter, sex, and weight. In no case were more than two animals from the same litter put on the same level of supplement. The supple-

mented food was eaten within 5 to 8 days and the unsupplemented ration was fed until 10 days had elapsed, counting from the beginning of the supplementary period. A record of the daily food intake was kept during the 10 day period, and if the rats lost weight or did not eat they were discarded.

After the animals were killed, the distal ends of the radii and ulnæ were removed and hardened in 10 per cent formalin for several hours. By means of the Shipley line test (7), the bones of the right wrist being used, the effect of the antirachitic supplement was determined.

Degrees of calcification were scored by means of plus and minus signs as follows:

No calcification in the metaphysis.....	—
Narrow broken line of calcification.....	±
“ continuous line of calcification.....	+
Medium line of calcification.....	++
Wide line of calcification or narrow epiphyseal cartilage ..	+++
Very narrow epiphyseal cartilage or complete healing....	++++

1 rat unit of vitamin D is defined as that amount of a substance which will produce a + cure.

RESULTS AND DISCUSSION

In Table I, the average daily food consumption and intake of units of vitamin D are recorded for the period 2 days to 29 days of age. Since the observations of each group were made under identical conditions and at the same time, it is of interest to note that daily fluctuations in food intake of one group are paralleled by those of the other in almost every instance. These data suggest that some environmental conditions may affect the food intake. The generally lower average food intake for the last 5 days of the experiment in the case of Group 2, and the resultant lower total food intake, are probably due to the poor condition of the bird that died on the last day of the experiment.

The weights of the two groups, Table II, were practically identical at 4 weeks of age. This similarity in weight at 4 weeks for both irradiated ergosterol- and cod liver oil-fed chicks has been observed in previous experiments in this laboratory. Although the percentage of bone ash for Groups 1 and 2 could not be determined

because it was necessary to extract the whole carcass, it could be assumed that the bone ash percentage of Group 2 was significantly higher than that of Group 1. It has already been noted in this laboratory (8) that at 4 weeks of age the percentage of bone ash of the pooled left tibias of White Wyandotte chicks supplied with 25 rat units of cod liver oil per 100 gm. of ration was 42 per cent, whereas that of birds fed 150 rat units of irradiated ergosterol per 100 gm. of food was 37.2 per cent.

The two ++ cures of Table III were not adequate to give quantitative results for the 48 hours old chicks. A perusal of Table IV, however, shows that ++ cures were produced by less than twice the 1 rat unit level. From the twenty-three cases of this kind in Table IV, it was concluded that 22 cc. of corn oil which contained the unsaponifiable residue from the 11 unfed 48 hours old birds were equivalent, at the most, to 4 rat units of vitamin D. Hess and his associates (9) reported that the livers of newly hatched chicks were practically devoid of the antirachitic factor and that the composite ethereal extract from the entire bodies of 50 newly hatched chicks was lacking in vitamin D when assayed with rats.

According to Table III no antirachitic factor was found in the concentrate which was equivalent to two irradiated ergosterol-fed chicks, and none in that equivalent to 1 cod liver oil-fed chick. Certainly the amount of the antirachitic factor was very small as compared with the number of units consumed. A further assay of the material from the 48 hours old and 4 weeks old chicks was not possible because the supply of concentrate was exhausted.

The results of the assay of the droppings of each group are given in detail in Table IV and summarized in Table V. For the unsaponifiable residue of the droppings of Group 1, 0.5 cc. of corn oil solution was chosen as 1 rat unit because this level produced the greatest number of + cures. In the case of Group 2, 1.2 cc. of corn oil solution were selected as 1 rat unit because the eight cures for this level were almost identical with the cures of the 0.5 cc. level of Group 1.

As shown in Table VI, only a part of the total number of units of the antirachitic factor ingested was recovered. A greater percentage of the antirachitic factor was obtained from the droppings of the growing chick when cod liver oil rather than irradiated

ergosterol was fed as a source of the factor, even though the latter was used in an amount biologically equivalent to more than 3 times the former. In addition to this fact, the actual number of rat units of irradiated ergosterol vitamin D not recovered is almost 3 times the number of rat units of cod liver oil fed and almost 5 times the number of units of cod liver oil not recovered. Hence if the units not recovered have been utilized by the chick, the data

TABLE V
Antirachitic Factor in Chicken Droppings

	Corn oil containing unsaponifiable residue from droppings	Volume of corn oil solution representing 1 rat unit	Per cent of total droppings representing 1 rat unit	Antirachitic factor in droppings
	cc.	cc.		rat units
Group 1, irradiated ergosterol.	170	0.5	0.29	340
Group 2, cod liver oil	180	1.2	0.67	150

TABLE VI
Summary of Antirachitic Factor Balance

	Antirachitic factor recovered from 11 unfed newly hatched chicks	Antirachitic factor ingested by 4 chicks in 4 wks.	Antirachitic factor recovered from the 4 wks old chicks	Antirachitic factor recovered from droppings		Antirachitic factor unaccounted for
	rat units	rat units	rat units	rat units	per cent	per cent
Group 1, irradiated ergosterol . .	4 at most	1285	None from 2 chicks	340	26.5	73.5
Group 2, cod liver oil	4 at most	348	None from 1 chick	150	43.1	56.9

suggest that antirachitic effectiveness is not associated with utilization of this factor.

Stability of the Antirachitic Factor

It is recognized that partial destruction of the antirachitic factor may have taken place during the process of the collection of the droppings and the preparation of the unsaponifiable extracts.

To test these possibilities the following five procedures were carried out.

A. Effect of Exposure of Irradiated Ergosterol with Chicken Droppings—A glass plate was placed under the pen of a group of chicks which were being fed a ration devoid of the antirachitic factor. Several hours later 2 cc. of corn oil containing 15 rat units of irradiated ergosterol were added to the droppings which had collected, and the plate was kept under the pen for 20 hours. The droppings were removed by means of a steel spatula and the

TABLE VII
Effect of Various Treatments on the Antirachitic Factor

Procedure	Line test scorings on levels of corn oil solutions used as follows:				Amount that should give + cure if no destruction	Degree of destruction
	1 cc.	1.5 cc.	2 cc.	3 cc.		
A		± ±	+ + ±		cc. 2	Slight destruction
B 1		± ±	± ±	± ±	2	" " (?)
B 2	+ ++	++ +++			1	No "
C 1		± —	++ +		2	" "
C 2	± +		++ ++		1	" "

glass plate was washed with a stream of ethylene dichloride. The mixture was stirred for a few minutes with CaCl_2 , filtered, and the residue on the filter paper was washed with 6 portions of ethylene dichloride.

B. Effect of Saponification and Extraction—(1) To ten livers devoid of the antirachitic factor 2 cc. of irradiated ergosterol solution, 15 rat units, were added. The livers were saponified and the saponified material was extracted with 300 cc. of ethylene dichloride. (2) 2 cc. of cod liver oil containing 46 rat units of

vitamin D were saponified and the hydrolysate extracted with 200 cc. of ethylene dichloride.

C. Effect of Storage with Ethylene Dichloride—(1) 2 cc. of corn oil containing irradiated ergosterol, 15 rat units, were added to ethylene dichloride and the solution kept in a refrigerator for 15 days. (2) 2 cc. of cod liver oil, containing 46 rat units, in ethylene dichloride were stored in a refrigerator for 18 days.

In all of the five procedures the ethylene dichloride was distilled off under diminished pressure, while the temperature of the water bath was between 60–70°. The residues of the irradiated ergosterol solutions were diluted with 30 cc. of corn oil, whereas those from the cod liver oil were diluted with 46 cc. of corn oil. Table VII displays the results of the assay of the five corn oil solutions.

The results, although qualitative in character, indicate that exposure with chicken droppings, cold saponification, extraction, and storage with ethylene dichloride all had but slight effect on the stability of the antirachitic factor. It is quite possible that the factor in the droppings on the glass plate during the balance experiment did not simulate the state in which it existed in Procedure A. The form in which this vitamin is excreted is not known. Although fed in oil solution, it is readily conceivable that, due to digestive processes through which the oil undergoes hydrolysis and absorption, the antirachitic factor may be excreted in the free state. Unless some protective agent was present in the droppings, the free antirachitic factor would be liable to suffer destruction between the time of excretion and storage in ethylene dichloride or even until stored in corn oil. The possibility is also recognized that all of the factor may not have been removed by the ethylene dichloride, although successive portions were used in the various extractions.

SUMMARY

1. Traces of the antirachitic factor were detected in the unsaponifiable residue from the bodies of unfed newly hatched chicks. The amount was less than 1 rat unit per chick.

2. No antirachitic factor could be found in the unsaponifiable residue of two 4 weeks old chicks which had been fed irradiated ergosterol, and none in that of one bird fed cod liver oil.

3. Of the total amount of the antirachitic factor fed as irradiated

ergosterol to chicks during the first 4 weeks of life, 26.5 per cent of the rat units consumed was recovered in the droppings. When cod liver oil was the source of the antirachitic factor, 43.1 per cent was recovered. The experimental data in each instance do not account for the remainder of the total number of rat units ingested.

4. Trials of a qualitative nature suggest that there was, at the most, only slight destruction of the antirachitic factor due to the processes of collection and concentration.

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THE GROWTH-PROMOTING PROPERTIES (VITAMIN B COMPLEX) OF THE CONCENTRATED WATER-SOLUBLE PORTION OF MILK

By G. C. SUPPLEE, O. J. KAHLENBERG, AND G. E. FLANIGAN

(From the Research Laboratories of The Dry Milk Company, Bainbridge, New York)

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The multiple character of the water-soluble growth-promoting factors has been well established by the researches of recent years (1-38), and even though only two specific factors are now universally recognized, namely vitamin B and vitamin G,¹ the existence of one or more additional factors is suggested by data from various sources.

While many of these studies have involved the use of yeast as the source of these vitamins, the presence of these factors in milk and other food products is generally conceded. Although many of the data regarding the relative abundance of the growth-promoting and antineuritic factors in milk are of an indirect character, the confirmatory evidence from various laboratories leaves no doubt as to their presence. Sherman and Axtmayer (26) have concluded from their studies on the supplementing effect of milk and grain that milk is richer in vitamin G than in vitamin B, whereas, in whole wheat, vitamin B is present in greater concentration than vitamin G. Outhouse *et al.* (29) have also submitted data indicating that vitamin B is relatively less abundant than vitamin G in cow's milk. Similar conclusions were reached by Hunt and Krauss (30). The classical work of Goldberger and associates (8, 9, 11, 12) based on field studies and laboratory experiments demonstrated the efficacy of milk in curing and pre-

¹ Vitamin B and vitamin G as referred to herein will be used in conformity with the recommendations of the Committee on Nomenclature of Vitamin B of the American Society of Biological Chemists, *Science*, **69**, 276 (1929).

venting pellagra in man and black tongue in dogs. Johnson and Hooper (39) found that it was necessary to incorporate as high as 30 per cent dry skim milk (spray process) in a polished rice diet in order to prevent polyneuritis in pigeons. This is equivalent to an intake of 6 to 7 gm. of milk solids, or about 75 cc. per day. Supplee and collaborators have recently obtained data (40) showing that a water-soluble vitamin concentrate prepared from milk possessed approximately 25 per cent of the antineuritic potency of rice polish, whereas, dried whey possessed only 10 to 12 per cent of the potency of rice polish, or slightly less than one-half that shown by the water-soluble concentrate. These data were obtained with pigeons, by using the curative technique. Norris, Heuser, and Wilgus (41), using the same product in experimental work with poultry, concluded that this concentrate contained another hitherto unrecognized factor necessary for the prevention of a particular type of paralysis in chickens and that it also promoted growth.

Inasmuch as the studies which will be reported hereinafter had as their primary objective the determination of the vitamin potency of certain water-soluble milk constituents, a suitable and readily adaptable method was a matter to be given first consideration. Since variations in the biological properties of the casein used in basal rations, even after supplementary purification, have been shown to exist (15, 31, 42, 43), our initial efforts were directed to the development of a method which would be satisfactory for determining variations in different samples of casein as well as for the study of the water-soluble milk vitamins.

EXPERIMENTAL

Part I. Variations in Biological Properties of Different Caseins

In considering the requirements of the method which it was desired to use, it was necessary to supply in the basal ration an amount of vitamin B just sufficient to prevent polyneuritis, and to maintain the animal at constant weight for long periods without permanent impairment of physiological functions. Such requirements imply the addition of a supplementing substance carrying a sufficiency of the maintenance factors in addition to the antineuritic vitamin. If these conditions could be met such growth response as resulted could be attributed to impurities of

the various caseins used, or, with a casein of predetermined purity, such a ration could be used for the assay of the growth-promoting factors in other products. Neither yeast, because of its reported variability in vitamin B content, nor its various fractions, as prepared by laborious means in different laboratories, seemed to be suitable for meeting the requirements at hand. Rice polish with its relatively high vitamin B content and apparently low vitamin G potency was considered as a possible supplement. In order to ascertain the merits of rice polish for the purpose under consideration a basal ration of the following composition was used: casein 20 parts, dextrin 60 parts, Crisco 10 parts, powdered agar-agar 2 parts, cod liver oil 2 parts, and Salt Mixture 40² 4 parts. To this basal ration were added varying percentages of a commercial grade of rice polish. The casein used for this series of tests was from a single lot taken from our stock supply, which had been purified some years previous by the following method:

A high grade commercial casein, precipitated by hydrochloric acid, thoroughly washed, and finely ground was leached with 0.1 per cent acetic acid for 10 days. 10 parts of the acidulated water was used for 1 part of dry casein; the leaching water was changed twice daily. Four washings with distilled water followed the acidulated water treatment, the proportions of casein and water being maintained as above. This casein will be designated hereinafter as Type A. Although no evidence has been revealed at this laboratory indicating the inadequacy of this procedure for satisfactorily freeing the casein of growth-promoting factors, the method became open to suspicion after the observations of Chick and Roscoe (15) and of Coward *et al.* (42, 43). These investigators showed that different samples of casein possess variable growth-promoting properties, and that these factors could be removed only after extraction with alcohol and ether following the treatment with acidulated water.

Typical results obtained by incorporating varying percentages of rice polish in the basal ration containing this particular sample of Type A casein are shown in Chart I. In this and in subsequent charts the growth curves of white rats are shown in conjunction with the mg. of daily intake of the test substance calculated on the weekly average basis.

² Steenbock, H., and Nelson, E. M., *J. Biol. Chem.*, **56**, 362 (1923).

The rats on the basal ration unsupplemented with rice polish usually died at the 5th to 6th week without substantial previous gain in weight. Animals receiving as high as 3 per cent rice polish did not show significant growth with this particular sample of casein. At the 4 and 7 per cent rice polish levels a slightly greater rate of growth resulted, although there was no substantial difference in growth at each of these levels. None of the animals receiving rice polish showed symptoms of polyneuritis. Practically all animals receiving 3 per cent or less of rice polish suffered loss of hair, dry skin, and not infrequently scaly lesions on the feet and tail. Extreme pellagrous symptoms as reported by other investigators have not been obtained with this diet. The conditions just described were usually evident at the 4th to 6th weeks. After a certain stage was reached further development of the pellagrous condition was rarely noted and in practically all instances the animal was maintained in substantially the same condition for several weeks. In the light of other investigations this reaction is possibly explained by the existence of a small trace of the antipellagric factor in the rice polish. This explanation is further strengthened by the fact that with the higher amounts of rice polish the pellagrous symptoms are less pronounced, and at the 7 per cent level they have been but rarely noted.

The results from this series of tests seemed to indicate the feasibility of incorporating 2 per cent rice polish in the basal ration as a supplement for supplying the minimum vitamin B and maintenance requirements. This ration has now been used for nearly 2 years for determining the relative amount of the water-soluble growth-promoting factors in various casein samples, miscellaneous products, and for the study of the milk vitamin concentrate with which this report is primarily concerned. It is desirable, however, to record that, although the casein used for the previously described tests was found to be free from accessory factors, later experiences showed that crude casein obtained under the specifications already mentioned and purified by leaching with acidulated water as described could not always be depended upon. Therefore, in order that the variability of different caseins may be better appreciated certain results which have been encountered are recorded at this time.

Chart I shows typical results obtained from another lot of casein procured under the same specifications as the lot used for the foregoing series of tests. The acidulated water purification

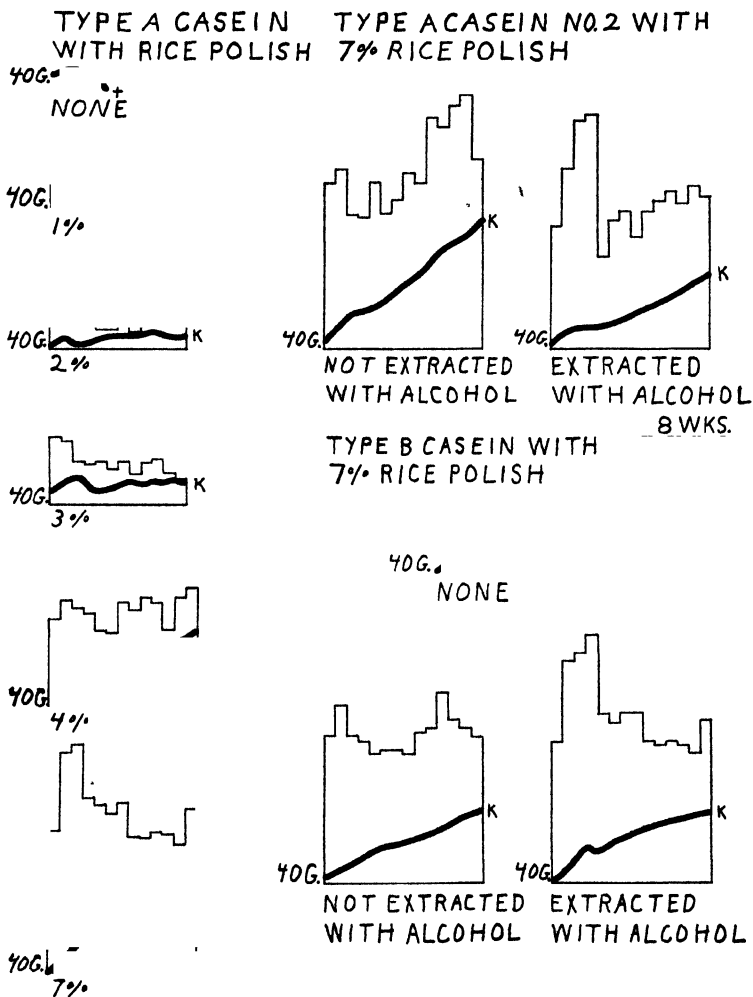


CHART I. Variations in the growth-promoting properties of different caseins when supplemented with rice polish.

procedure was carried out in the manner previously noted. The rice polish was from the same lot previously used. It is to be observed that the degree of growth resulting from the use of this casein supplemented with 7 per cent rice polish was substantially greater than that obtained from the previous lot. This casein after alcohol and ether extraction for a period of 48 hours each gave substantially less growth than before extraction.

Examples of the results obtained from another lot of casein are also shown in Chart I. This was a commercial product manufactured by the "cooked curd" process, wherein the casein is heated in the whey to a sufficiently high temperature after precipitation with acid to cause coalescence of the curd particles. This type of commercial casein, designated as Type B, was received in a dry, finely ground condition and subjected to the usual purification procedure. This casein gave unsatisfactory results even when unsupplemented with rice polish. It is to be noted that the results are substantially the same, when supplemented with 7 per cent rice polish, before and after alcohol and ether extraction. Failure to obtain greater growth in the presence of 7 per cent rice polish might indicate, in the light of present knowledge, that the impurity in this particular casein was primarily vitamin B.

The results from these tests appear fully to confirm the observations of other investigators to the effect that the acidulated water treatment cannot always be relied upon completely to free all commercial caseins from the growth-promoting factors. These results also indicate the possibility of a selective adsorption and retention of the water-soluble factors by casein prepared by different methods. The meager evidence available accentuates the importance of further basic studies regarding the biological character of caseins used in the basal rations required for the determination of certain vitamins.

Part II. Growth-Promoting Properties of the Water-Soluble Portion of Milk

Since the publication of a brief preliminary report indicating the nutritive character of the concentrated water-soluble portion of milk, further data have been obtained. The product under consideration represents a concentration of milk constituents

other than the fat, casein, albumin, and lactose, and since there is no evidence which indicates a direct correlation of the water-soluble vitamins with these substances, other than a mechanical

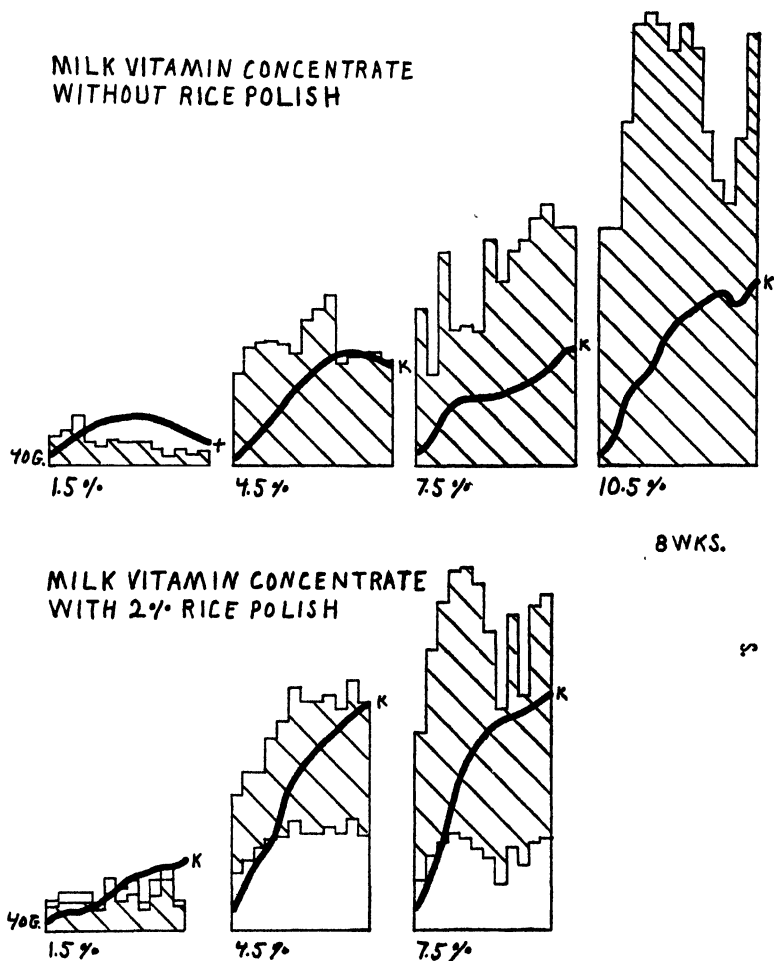


CHART II. Growth-promoting properties of the water-soluble milk vitamin concentrate with and without rice polish.

one, it is logical to believe that these factors and other important dietary accessories found in natural milk are contained in the concentrate with which these studies are concerned.

The gross composition of the concentrate used for these tests, calculated to the dry basis, follows: lactose 52.01 per cent, ash 30.84 per cent, and nitrogen 1.54 per cent. This product was incorporated in the test rations in the desired proportions by drying on predetermined amounts of dextrin. The basal ration containing casein of predetermined purity was the same as given in "Part I" of this report.

White rats reared in our own stock colony were selected for use at 40 to 50 gm. of weight, preferably between 40 and 45 gm. of

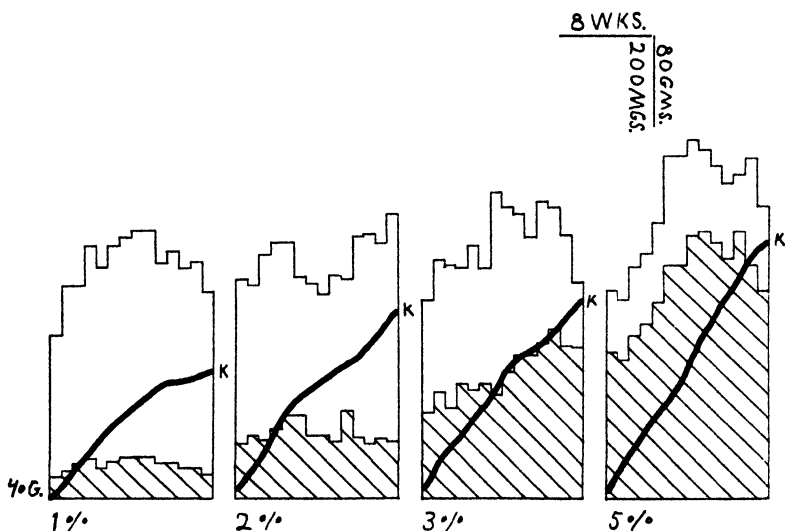


CHART III. Growth-promoting properties of the water-soluble milk vitamin concentrate supplemented by 7 per cent rice polish.

weight. All animals were kept in individual wire cages with screen bottoms. Food consumption records were uniformly maintained, and for convenience and clarity the daily average intake of the supplementing test substances is shown in graphical form on the accompanying charts.

Chart II shows typical results obtained from varying percentages of the water-soluble milk vitamin concentrate with and without 2 per cent rice polish supplement. Those animals receiving 1.5 per cent of this concentrate not only failed to grow but manifested subacute symptoms of polyneuritis a few weeks before

death. These symptoms were not manifested by those animals receiving the higher amounts. It is to be noted that the milk concentrate unsupplemented by the vitamin B of the rice polish is inadequate for normal and continued growth at a daily average intake of approximately 250 mg., but that normal growth does result when a daily average intake of about 900 mg. is reached. When the basal ration was supplemented with 2 per cent rice polish normal growth was maintained at a daily average intake of

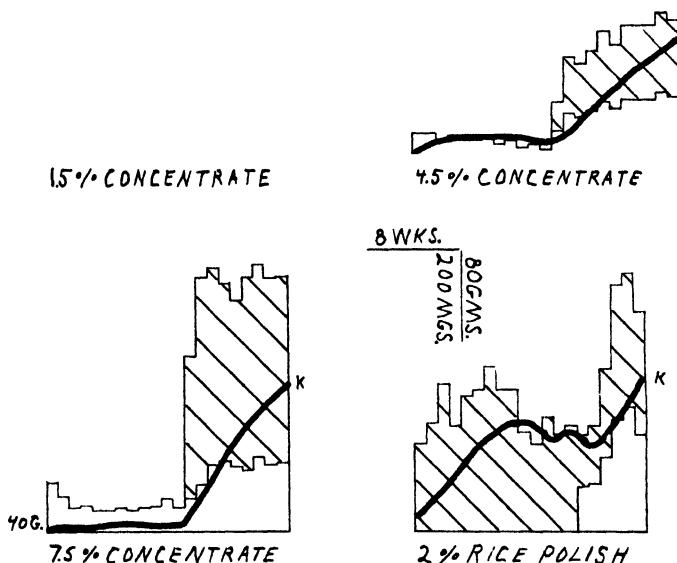


CHART IV. The effect of the water-soluble milk vitamin concentrate and rice polish as subsequent supplements following suppression of growth.

about 400 mg. All data show that the vitamin B potency of this concentrate is relatively lower than its vitamin G potency. Additional data are also shown in Chart III wherein the rice polish supplement is increased to 7 per cent. With this higher amount of rice polish substantially normal growth was maintained at a daily average intake of between 100 and 200 mg. of the milk vitamin concentrate.

These results show that a comparable growth response may be obtained from varying proportions of each of the different factors

concerned, and that while vitamin B as well as other factors, must be present, fixed proportions or relationships between each are not necessarily a prerequisite for maintaining a particular rate of growth.

In order further to determine the growth-promoting and anti-pellagic properties of the water-soluble milk vitamin concentrate, varying percentages of this product were superimposed on the basal ration containing 2 per cent rice polish, as a subsequent sup-



FIG. 1. Rat 2842 after 13 weeks on basal ration supplemented with 2 per cent rice polishings. Weight 43 gm.



FIG. 2. Rat 2842 3 weeks after receiving the water-soluble milk vitamin concentrate furnished at the 13th week (Fig. 1). Weight 122 gm.

plement following suppression of growth; rice polish was also furnished as a subsequent supplement after a decline in weight resulting from the feeding of limited amounts of the concentrate. Chart IV shows illustrative data from this series of tests. During the period of constant weight the mild symptoms of pellagrous character previously described were manifested. None of the animals showed symptoms of polyneuritis. At the 1.5 per cent level the growth response was slight and the pellagra-like condition was somewhat improved. At the higher levels growth was resumed

at a rapid rate and the pellagrous symptoms began to show improvement within 3 to 5 days. The complementing effect of rice polish and the milk vitamin concentrate was further illustrated by the prompt growth response brought about by 2 per cent rice polish furnished as a subsequent supplement after decline in weight resulting from limited amounts of the concentrate.

Fig. 1 shows the typical condition of the animals just before the beginning of the subsequent supplementary feeding of the milk concentrate. The weight of Rat 2842, at the beginning of the experimental feeding period was 40 gm. and after 13 weeks during which time it received the basal ration supplemented with rice polish only, its weight was 43 gm. Fig. 2 shows the same animal 3 weeks later at a weight of 122 gm. after it had received a subsequent supplement of 7.5 per cent of the water-soluble milk vitamin concentrate. A similar growth-promoting potency of this substance has since been demonstrated with hundreds of animals. A sample of the concentrate further purified but still containing much inert material has caused a growth response after suppression of growth of as high as 59 gm. per week.

In view of the growth response shown by animals receiving varying proportions of the known or suggested accessory factors, the potential powers for utilization of these accessories during different periods of life raise the question of the specificity of the growth-promoting vitamins. These relationships clearly indicate that nutritional studies dealing with the question of vitamin specificity should take into consideration not only the specific factors but also the vitamin balance of the dietary constituents and the ability of the animal to use the factors existing in such balance. A natural corollary to these considerations is the question of the optimum interrelationship of accessory factors which will permit normal development and continued well being without permanent impairment or disturbance of physiological processes.

Part III. Effect of High Temperatures and Ultra-Violet Radiations on the Water-Soluble Milk Vitamin Concentrate

The growth-promoting properties of the milk vitamin concentrate with and without a supplement of rice polish having been determined, the stability of these properties at high temperatures

and under intensive ultra-violet ray irradiation was investigated. Similar methods of experimentation have been used to study the

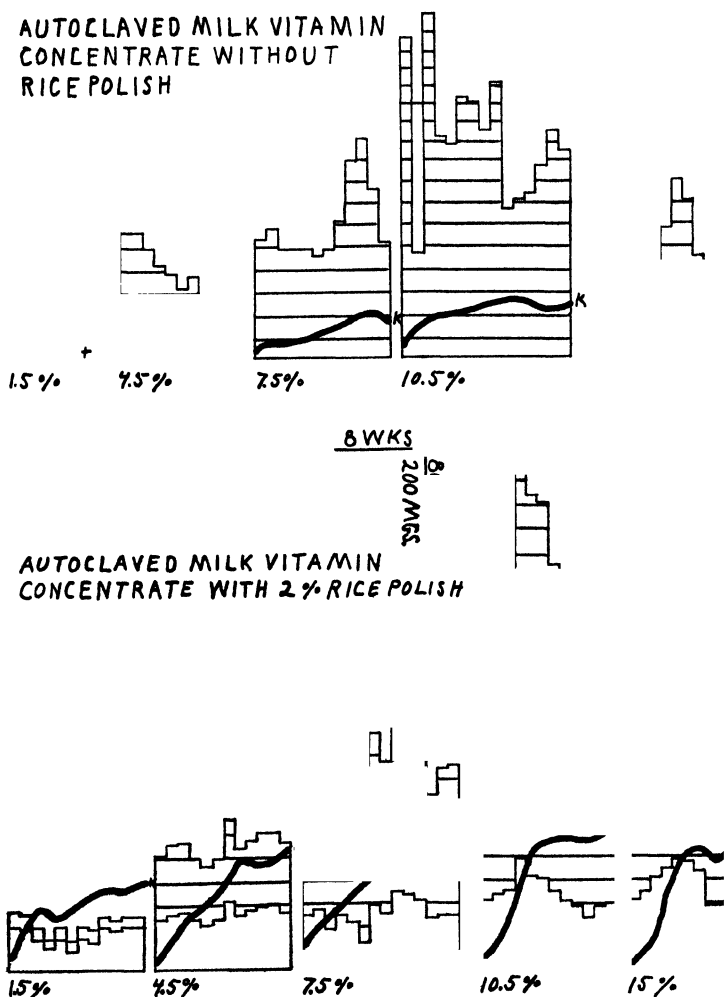


CHART V. Results obtained from the water-soluble milk vitamin concentrate after autoclaving.

multiple character of the water-soluble vitamins of yeast (10, 11, 20, 22, 24-26, 37).

After the milk vitamin concentrate was adjusted to pH 9 it was autoclaved for 5 hours at 120° during which time the pH dropped to 4.5. The autoclaved material was fed with the basal ration unsupplemented, and also supplemented with 2 per cent rice

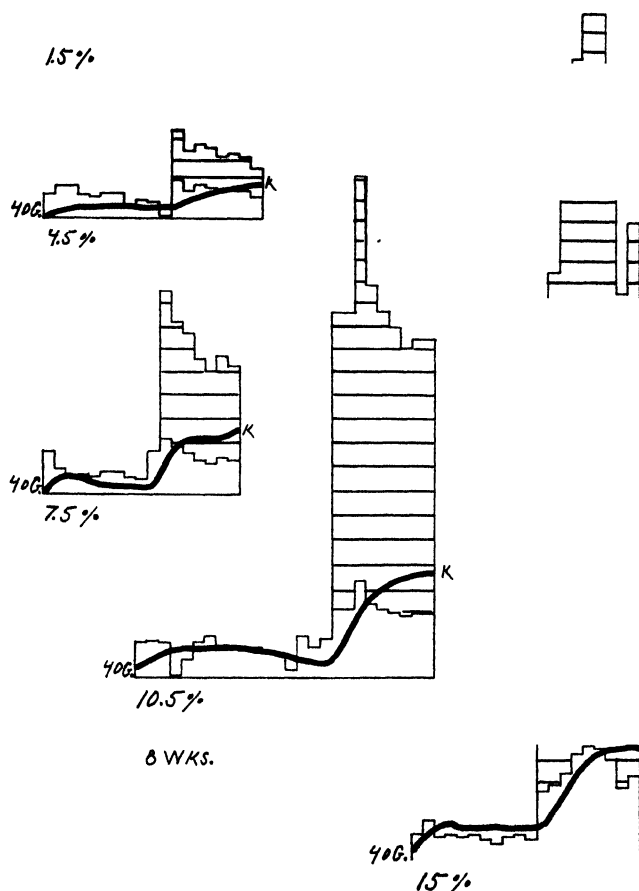


CHART VI. Results obtained from the autoclaved water-soluble milk vitamin concentrate furnished as a subsequent supplement.

polish, at levels varying from 1.5 to 15 per cent from the beginning of the feeding period, and also as a subsequent supplement after suppression of growth and development of mild pellagrous symptoms. Typical results are shown in Charts V and VI.

A lower rate of growth, as compared with similar quantities of unheated material, was shown by all animals, irrespective of the amount of test substance ingested. For the groups receiving the autoclaved material supplemented with 2 per cent rice polish from the beginning of the feeding period (Chart V) the rice polish intake was substantially the same with the exception of that of the groups receiving the 1.5 per cent level. In practically all these instances a notch, flattening, or decline of the growth curve appears at a period, or at a stage of development, which substantially coincides with the period of adolescence. In those groups receiving the autoclaved material at the higher levels after suppression of growth the same phenomena are indicated. Pellagrous symptoms were improved in those animals which resumed growth. Those receiving the lower amounts and which did not resume growth showed no improvement in these symptoms. The fact that the daily average intake of the autoclaved concentrate varied from approximately 300 mg. per day to as high as 1400 to 1500 mg. without causing a commensurate growth response in the presence of a relatively constant rice polish intake, indicates in comparison with the data previously recorded, that vitamin G as it exists in milk and as now defined, involves more than a single entity.

These data clearly indicate the thermolabile character of one or more of the growth-promoting factors contained in the water-soluble milk vitamin concentrate. The evidence also suggests that the thermolabile factor or factors which exist in this product are utilized by the animal organism particularly at and following the period corresponding to that of sexual maturity. The evidence is such that it cannot be assumed that this factor is vitamin B (antineuritic vitamin) as now defined in its restricted sense, or, if the antineuritic vitamin alone is concerned a specific demand at and beyond the period of adolescence is indicated. The results again emphasize the question of the specificity of the various water-soluble factors in so far as growth requirements are concerned. They likewise bring into prominence the particular requirements of the animal for these factors during different periods of the life cycle. The evidence now available might be interpreted to mean that the requirement for such factors during the period of structural growth is relatively lower than during adolescence.

Conflicting data have been reported (20, 24) in regard to the destruction of the water-soluble factors by ultra-violet rays. This subject has been given consideration in connection with our studies on the milk vitamin concentrate. The concentrate was irradiated in water solution at 10 per cent concentration, at a distance of 12 inches from a quartz mercury vapor lamp for 10 hours in a layer approximately 2 mm. thick. The material was agitated at $\frac{1}{2}$ hour intervals; water was added as required to replace that lost by evaporation. The irradiated material was fed at various levels supplemented and unsupplemented with 7 per cent rice polish.

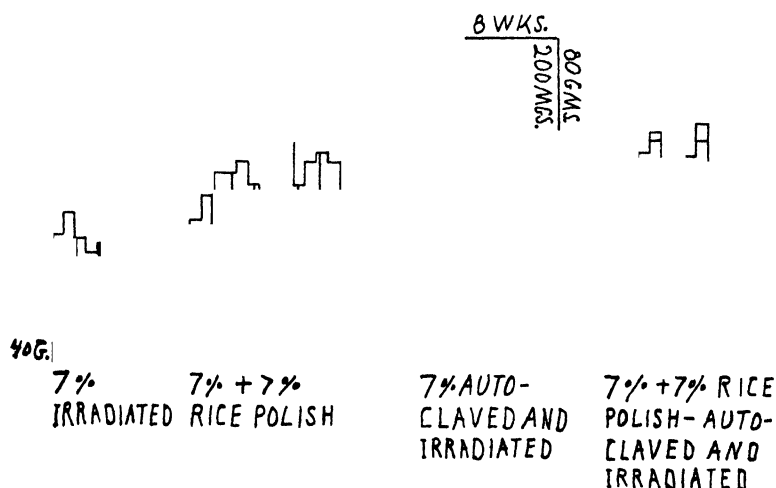


CHART VII. Results obtained from the irradiated and autoclaved water-soluble milk vitamin concentrate.

Certain groups also received autoclaved material as a further supplement. Typical results as recorded in Chart VII show that ultra-violet rays intensively applied as under the conditions of this test have a destructive effect upon vitamins B and G. The animals receiving 7 per cent of the irradiated material showed acute symptoms of polyneuritis before death. Those receiving the same amount of irradiated concentrate supplemented with 7 per cent rice polish showed no greater growth than the animals receiving the same amount of rice polish alone. The animals receiving the concentrate which had been both irradiated and autoclaved provide further evidence of the destructive effect of

intensive irradiation on the growth-promoting factors. Several similar series of experiments were made wherein different lots of irradiated material were fed at different levels. Inconsistencies in the destructive action of the ultra-violet rays on the growth-promoting properties were observed. Such results indicate that irradiation as applied under the particular conditions of these experiments cannot always be relied upon completely to destroy

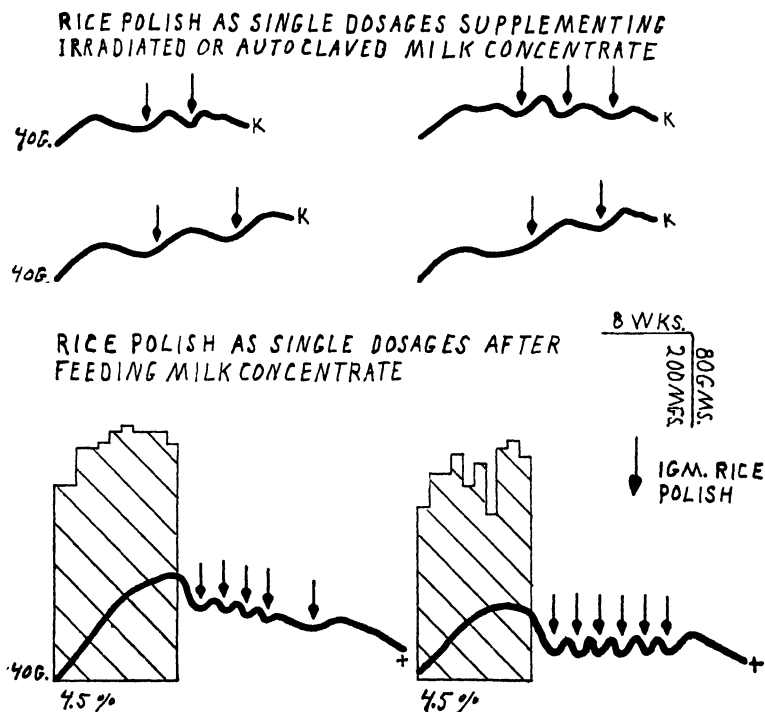


CHART VIII. Results obtained from single doses of rice polish

the growth-promoting factors. The production of toxic substances by irradiation under these conditions does not appear to have been responsible for the irregularities observed. In one series of tests wherein as high as 14 per cent of the irradiated and autoclaved concentrate was fed, the animals maintained substantially constant weight for a period of 8 weeks. At this point 3.6 per cent of non-irradiated and unheated concentrate was superimposed as

a further supplement. Growth response was prompt, practically all animals gaining at the rate of 15 to 18 gm. per week during the next 5 to 6 weeks. Since the effect of radiant energy of this character is dependent upon the penetrability of the particular material to the rays under consideration, ineffective and irregular results may be obtained as a result of purely mechanical considerations.

Further evidence of the destructive effect upon vitamin B and the growth-promoting factors contained in the milk vitamin concentrate by autoclaving and irradiating is shown in Chart VIII. The graphs represent composite records from thirteen to twenty-seven animals. These results were obtained by feeding variable amounts of the irradiated concentrate supplemented by varying amounts of non-irradiated autoclaved material. In all instances subacute polyneuritis was first observed between 8 and 10 weeks. When these symptoms were first manifested, 1 gm. doses of rice polish were given. The dose was usually consumed within 6 to 12 hours. Improvement in the polyneuritic condition followed the rice polish dosage, such improvement being accompanied in all instances by a slow gain in weight extending over a period of from 1 week to 10 days. Decline in weight then followed over a somewhat irregular period. Upon reoccurrence of polyneuritic symptoms 1 gm. doses of rice polish were again given with repetition of the reactions just described. Chart VIII also shows the records of two of eight animals indicating the effect of single doses of rice polish on mature animals after complete withdrawal of the growth-promoting factors of the unheated and non-irradiated milk vitamin concentrate from the basal ration. In the absence of the accessory factors furnished by the concentrate a rapid decline in weight resulted with early manifestation of polyneuritis. At the first occurrence of these symptoms 1 gm. doses of the milk vitamin concentrate were given without checking the decline in weight or without perceptibly alleviating the polyneuritic condition. 1 gm. doses of rice polish were subsequently fed, resulting in prompt improvement of the polyneuritis symptoms and resumption of growth. After about 1 week, decline in weight again took place accompanied by prompt reappearance of the polyneuritic condition. 1 gm. doses of rice polish were fed on reoccurrence of these symptoms with a high degree of regularity in repetition of the results just described. These results would

seem to show clearly the specificity of rice polish as a carrier of the antineuritic vitamin as well as to indicate the correlation of this vitamin with other factors necessary for the promotion of growth.

SUMMARY

Data are presented confirming the investigations of other investigators to the effect that acidulated water treatment cannot always be relied upon completely to free all commercial caseins from the water-soluble growth-promoting factors. The results also indicate the possibility of a selective adsorption and retention of the different factors of the vitamin B complex by caseins prepared by different methods. The meager evidence indicates that further studies are needed on the biological character of caseins used in the determination of certain vitamins.

Limited amounts of rice polish supplementing a suitable basal ration were found to supply white rats with sufficient amounts of the water-soluble maintenance factors to permit comparable assays of the growth-promoting vitamins. Assurance of the purity and suitability of particular lots of casein is prerequisite for determinations of this character.

The concentrated water-soluble fraction obtained from milk after the removal of the fat, casein, albumin, and a substantial proportion of the lactose, is shown to contain a high concentration of the growth-promoting and antipellagic factors. This concentrate contains less of the antineuritic principle (vitamin B) than of the other water-soluble growth-promoting factors. The growth response resulting from limited amounts of this milk derivative is increased when additional amounts of vitamin B as found in rice polish are supplied.

Growth induced by the milk vitamin concentrate, either unsuppressed or resumed after suppression, concurrently prevented or alleviated the pellagic symptoms noted in these studies.

Growth may take place even though there are wide variations in the proportions of the accessory factors supplied by the water-soluble milk vitamin concentrate and rice polish. The results indicate the unreliability of predictions as to the amount of any one factor required for normal development, without knowledge of the relative amount of the other factors present; all of the known factors must be present to some degree.

The autoclaving of a water-soluble milk vitamin concentrate at 120° for 5 hours adversely affects its growth-promoting properties.

Intensive irradiation with ultra-violet rays for a period of 10 hours adversely affects the growth-promoting properties of the milk vitamin concentrate. Such treatment, however, cannot always be depended upon completely to destroy the growth-promoting factors. Data are presented which indicate that this irregularity may be due primarily to the inefficiency of the irradiation technique.

Data are presented indicating that accessory water-soluble factors other than vitamins B and G as now defined may be required during different periods of the life cycle, particularly at and following the period of adolescence. Final conclusions as to the existence of such additional factors must await further knowledge of the specificity of the water-soluble factors and the ability of the animal to utilize such factors during the different periods of development.

Such factors as may be concerned in the maintenance of normal development through the period of structural growth as well as during the period of adolescence and beyond, and irrespective of their final identity and classification are, according to these studies, present in the water-soluble fraction of milk. Data are presented indicating that such factors are thermolabile at the high temperatures used in these experiments.

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**A SOURCE OF ERROR IN NITROGEN AND PHOSPHORUS
DETERMINATIONS ON FILTRATES OBTAINED AFTER
PRECIPITATION OF TISSUE COLLOIDS BY
TRICHLOROACETIC ACID OR OTHER
STRONG ACID**

By H. D. KAY

*(From the Department of Biochemistry, University of Toronto, Toronto,
Canada)*

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Trichloroacetic acid is now very widely used for the precipitation of tissue proteins as a preliminary to the analysis of several of the tissue constituents that come through unchanged into the protein-free filtrate. Its use as a precipitant has many advantages among which may be numbered the completeness of precipitation, the quantitative precipitation of lipids as well as proteins by it, the ready filtrability of the precipitates, the freedom of the commercial product from serious impurities and its easy purification by redistillation if desired, and the ease with which determinations of nitrogen, phosphorus, inorganic salts, glucose, lactic acid, and many other substances may be made in filtrates containing trichloroacetic acid.

It is, however, a strong acid, and certain tissue constituents are well known to be unstable to the concentrations of this acid which are required for protein precipitation. A typical example of such a tissue constituent is the phosphagen in muscle (Eggleton and Eggleton (1); Fiske and Subbarow (2)). Another compound, rather more stable to it, is pyrophosphate, discovered by Lohmann (3) to be present in muscle probably in organic combination. Pyrophosphoric acid and its esters are slowly decomposed by fairly strong acids at room temperatures, and much more rapidly at 37°.

It is not so generally appreciated that at least one other hydrolytic reaction occurs, though not so rapidly, if tissues are treated with trichloroacetic acid or other strong acid and filtration does not follow immediately. In some recent experiments in which a

large number of determinations were being carried out with the tissues of rats immediately after death, the proteins of liver tissue were precipitated by 5 per cent trichloroacetic acid. Owing to press of other analyses, the precipitate was allowed to remain in contact with the acid fluid for over 24 hours before filtration.

TABLE I

Effect on Inorganic and Total Phosphorus Present in Filtrate of Keeping Precipitate in Contact with Trichloroacetic Acid

The tissue was ground up with 8 times its weight of 5 per cent trichloroacetic acid.

Tissue	Filtered, time after precipitation	P in filtrate, mg per 100 gm. tissue		
		Inorganic	Total	Organic
	<i>hrs.</i>			
Liver (rat)	0	46	116	70
	20	44	156	112
	44	45	171	126
	92	44	210	166
Brain (rabbit)	0	45	76	31
	1	46	80	34
	6	45	95	50
	40	45	163	118
	91	45	199	154
Muscle (rat)	0	54	106	52
	4	54	108	54
	24	55	118	63
	48	54	135	81
	96	55	136	81
Plasma (dog)	0	4.9	5.2	0.3
	48	5.0	6.2	1.2
	144	4.9	8.5	3.6
	168	4.8	8.2	3.4

It was found on analyzing the filtrate that, while the inorganic phosphate was normal in amount, the *phosphoric ester* value in the filtrate was considerably higher than was anticipated. On looking into the matter it was observed that the rise in phosphoric ester could be correlated with the time elapsing between pre-

cipitation and filtration. Experiments showing the magnitude of this effect for liver and for other fresh tissues are given in Table I.

It was soon realized that an effect of this magnitude could only come, at least in plasma, from slow hydrolysis of lipids in the protein + lipid precipitate, with the slow liberation of glycerophosphoric acid and of the soluble nitrogen-containing moiety of the lipid molecule.

This was confirmed by experiments with "synthetic" tissue. Samples of pure egg albumin and pure lecithin were dissolved in water and mixed with a suitable salt solution. Trichloroacetic acid to 5 per cent strength was then added, and phosphorus deter-

TABLE II

Rate of Breakdown of Pure Lecithin in Artificial Tissue Precipitated by Trichloroacetic Acid

53.3 mg. of organic P and 24.1 mg. of lecithin N per 100 cc. of reaction mixture.

	P, mg per 100 cc. filtrate			N, mg per 100 cc. filtrate	N:P ratio
	Inorganic	Total	Organic		
<i>days</i>					
0	6.3	8.3	2.1	11.1	
3	6.2	32.4	26.2	22.2	1:1.02
10	6.2	52.0	45.8	31.2	1:1.02
				Theory for lecithin = 1:1	

minations carried out on the filtrate at intervals. The only possible source of phosphoric ester was now the lecithin. Determinations of the total nitrogen in the filtrates, both from the synthetic tissue and from rat liver, showed that there was a slow increase in nitrogen, proportional to the increase in organically bound phosphorus, if the precipitate were allowed to remain in contact with the acid solution (Table II).

Figures like those in Table II are given when fresh tissues are treated similarly. The nitrogen increase in the filtrate is roughly proportional to the increase in the organic phosphorus, while the inorganic phosphorus value remains constant. Here, however, as might perhaps be expected, the N:P ratio in the filtrate does not point to the slow hydrolysis of a monoaminomonophosphatide

only, but probably represents the sum of several more or less complex reactions which may involve other compounds in addition to the tissue lipids.

The same type of slow hydrolysis also occurs when the well known Schenck method ($\text{HgCl}_2 + \text{HCl}$) of protein precipitation is employed. An example may be briefly quoted. Liver tissue was ground up with mercuric chloride and hydrochloric acid added. Samples of the mixture (which had a total HCl content of 0.5 per cent) were filtered after standing for 0 and 17 hours and phosphorus and nitrogen determinations made on the filtrates. There was an increase in phosphoric ester in the filtrate from 30 to 74 mg. and of nitrogen from 96 to 215 mg. (calculated per 100 gm. of fresh liver tissue) as a result of 17 hours standing.

There is thus a fairly rapid liberation of some nitrogen compound and also of a phosphoric ester from the precipitate into the liquid after it is allowed to stand for a short time with the Schenck reagents. (When this method is used, precipitate and supernatant liquid are, in practice, frequently allowed to stand together overnight before filtration.)

These findings recall an observation of Lüdecke (4) who noted in 1905 that lecithin was slowly decomposed on standing with picric acid, even at room temperature. This observation has been half forgotten. But it is very evident from the work reported here that any findings dependent on the determination of phosphoric ester or nitrogen or choline, or even amino nitrogen (from the aminoethyl alcohol of cephalin), in the filtrate from precipitated tissue colloids may be vitiated if the precipitated colloids have been allowed to remain in contact with the acid precipitant for more than a very limited period. In particular, claims as to the presence of free choline or phosphoric esters in tissues submitted to acid precipitation require scrutiny. Thus a statement was made by me (5) that a relatively minute amount of an acid-soluble phosphoric ester, hydrolyzable by tissue phosphatases, is present in blood plasma. Trichloroacetic acid had been used to precipitate the plasma proteins before proceeding with phosphorus determinations in the filtrates, and the precipitate had been left in contact with the acid liquid for some minutes at least before filtration.

The presence or absence of phosphoric esters from plasma has considerable bearing on the theory that a phosphatase plays some part in bone formation and maintenance, and, in order to re-examine this point, experiments have been carried out in which relatively large quantities of plasma, freed from red blood cells and leucocytes, have been precipitated by trichloroacetic acid, and determinations of inorganic and total acid-soluble phosphorus made on samples of this mixture, filtered at once and at increasing intervals of time after precipitation. It was found, however, that there is little if any increase in either the inorganic or the organic phosphorus of the filtrate from trichloroacetic acid precipitation of plasma if the filtration is carried out within half an hour. One example, typical of several, with, in this case, freshly separated dog plasma, gave the following figures for ester phosphorus in the filtrate (calculated for 100 cc. of plasma): filtered at once after precipitation 0.24 mg., filtered after $\frac{1}{2}$ hour 0.3 mg., filtered after 3 hours 0.6 mg., filtered after 24 hours 1.3 mg.

In the case of liver tissue, certain properties of the phosphoric ester liberated by allowing the trichloroacetic acid precipitate to stand for a week in contact with the acid liquid confirm the opinion that it is a glycerophosphate; *e.g.*, its stability to 0.25 N NaOH at 98°, the solubilities of its barium and lead salts, its ready hydrolyzability by kidney and by taka-phosphatases. There is some evidence from the rate of hydrolysis by taka-phosphatase that this glycerophosphate may contain a fairly high proportion of the α -isomer.

SUMMARY

If it is desired to determine the non-protein nitrogen, choline, amino nitrogen, or acid-soluble phosphoric ester content of a fresh tissue, and a method of deproteinization is employed which entails the use of a strong acid (*e.g.* the trichloroacetic acid method, the Schenck method), it is essential that the precipitate should not remain in contact with the acid for more than a few minutes. If this precaution is not taken, particularly with tissues having a high lipid content, there is the certainty of hydrolysis of lipids, which may proceed sufficiently far in a short time to render the results of such determinations entirely without value.

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THE PHOSPHATASES OF MAMMALIAN TISSUES

III. MAGNESIUM AND THE PHOSPHATASE SYSTEM

By H. D. JENNER AND H. D. KAY

(From the Department of Biochemistry, University of Toronto, Toronto, Canada)

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Erdtman (1) in 1927 made the discovery that on prolonged dialysis kidney phosphatase preparations slowly lost much of their activity, part of which could be restored by adding the boiled concentrated dialysate. He found that the activator was also present in meat extract, and finally came to the very interesting conclusion that the activator was almost certainly Mg ions which in relatively low concentrations were effective in increasing the activity of dialyzed phosphatase preparations. Calcium and beryllium had no great influence on the activity, and zinc was somewhat inhibitory. This work was pursued by Hommerberg (2), who believed that the effect of magnesium on bone phosphatase was different from that on kidney phosphatase. He found, in fact, that the addition of magnesium caused inhibition of the bone enzyme, while it caused powerful activation of the kidney phosphatase. This would indicate a fundamental difference between the two enzymes, and might be of importance in the consideration of their normal functions in the body.

In the present paper the function of magnesium as a specific activator for various tissue phosphatases has been examined more closely.

Enzyme Preparations Used—Three or four methods have been recommended for the purification of phosphatase, and these may in fact yield preparations of a somewhat higher activity per unit weight of dissolved or suspended solid than extracts of fresh tissue. However there is in all the methods recommended so far, a very serious loss of enzyme activity in the "purification" process without any very definite compensations. In the majority

of the experiments to be recorded, the enzyme has been extracted from the ground tissue by chloroform water along the lines previously described (3), entailing relatively little loss of the enzymic activity of the tissue, and then either dialyzed or electrodialyzed for differing periods, and filtered through a coarse filter. The enzyme preparations have been made mainly from kidney, intestinal mucosa, and bone, and a few from liver. Red blood cell phosphatase and plasma phosphatase have also been investigated. Various animals, including cats, rats, rabbits, dogs, and man, have supplied the tissues.

Buffers—In the majority of the experiments where buffers have been required Sørensen's glycine-NaOH buffers have been used.

Substrate—A solution of pure crystalline sodium β -glycerophosphate has been employed in the majority of experiments. In a few instances other phosphoric esters have been used.

Optimal Concentration of Mg for Activation of Phosphatase

In dealing with the rather small concentrations of Mg required for activation of phosphatase the same awkwardness of notation arises as was frequently encountered in dealing with hydrogen ion concentration in pre-pH days. Concentrations, say, of 0.00023 M Mg are not only awkward to express in this longhand way, but in many cases have to be converted into logarithms before they can be most advantageously plotted. However, to use the Sørensen method of notation, $p\text{Mg}$, to express the negative logarithm of the Mg ion concentration (in normality) suggests either that the Mg is present entirely as ions or that electrode measurements of Mg ion have been made, both alternatives being misleading. It was thought therefore that it would be most useful and easily understood if some other symbol were used to denote concentrations, namely $q\text{Mg}$, the negative logarithm of the Mg concentration expressed as *molarity*; e.g., $q\text{Mg}$ of 2.70 = 0.00200 molar Mg, etc.

It was found in preliminary experiments that the optimal concentration of Mg lay in the region of $q\text{Mg}$ 1 to 3 for almost all the tissue phosphatases used. In the case of one tissue phosphatase—that of the plasma—a figure has already been reported by one of us (4), namely $p\text{Mg}$ of 1 to 2 or $q\text{Mg}$ of 1.3 to 2.3. If these limits are extended from $q\text{Mg}$ 0.94 to $q\text{Mg}$ 3.4, they include all the optima

in some two score experiments that we have made. The majority of the experiments show a more or less broad $q\text{Mg}$ optimum between 1.7 and 3.3. An illustration of the type of result we obtain is given in Fig. 1, which at the same time shows that whether the Mg is added as chloride or sulfate the shape of the $q\text{Mg}$ -activity curve is very little affected. Glycine buffers at pH 8.9 (about the optimal pH for animal phosphatases) were used, the magnesium usually was added as MgCl_2 (adjusted to pH 8.9) and the incubation period was usually 2 hours at 37.5° .

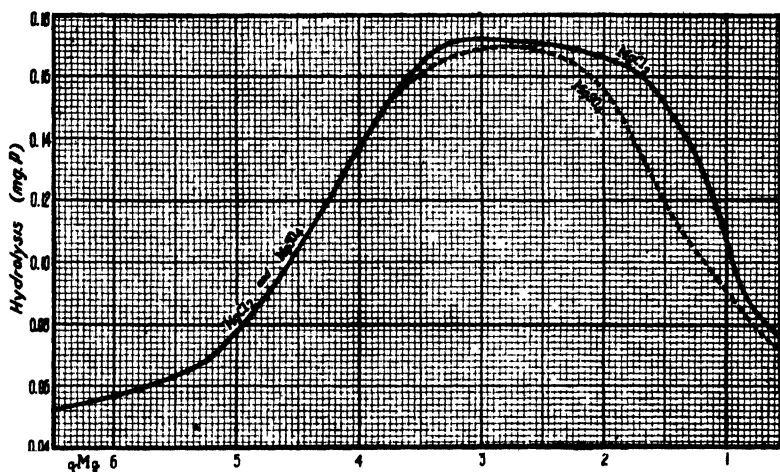


FIG. 1. The $q\text{Mg}$ -activity curve for the action of dialyzed kidney phosphatase on sodium β -glycerophosphate at pH 8.9 in the presence of increasing quantities of Mg. ($q\text{Mg}$ = the negative logarithm of the molarity of Mg in the reaction mixture.)

We may state our findings as follows:

Nature of Tissue from Which Enzyme Is Derived—The origin of the phosphatase (derived from various tissues or organs of a number of different mammals) does not have any marked effect on the position of the broad optimum. The source of the enzyme has, however, some effect on the *extent* of activation by magnesium. Intestinal preparations exhibit on the whole a greater degree of inactivation during dialysis and a greater degree of stimulation following the addition of Mg to the dialyzed preparation than either kidney or bone preparations. Some red blood cell prepara-

tions show as much as 20-fold activation by Mg, even without dialysis. The extent of activation may possibly reflect the minuteness of the amount of Mg remaining in the dialyzed (or even in the untreated) tissue extract (but see the following paragraphs).

Extent of Dialysis—As first observed by Erdtman, we found that as dialysis proceeded the enzyme preparation became less and less active, but despite prolonged simple dialysis in collodion sacs against water, sodium chloride solutions, and against buffers at various pH (in the hope of replacing possible traces of Mg adsorbed on the enzyme or associated with it in some type of salt-

TABLE I
Effect of Simple Dialysis through Collodion on Activation of Phosphatase by Mg

Source of phosphatase	Activity in phosphatase units per cc. of extract		Activity after adding Mg (original activity = 100)
	Without Mg	At optimal qMg	
Kidney undialyzed	1 35	1.54	114
Same preparation dialyzed 14 days at 2°	0.49	1.38	280
Intestine undialyzed	2.20	3.63	165
Same preparation dialyzed 14 days at 2°	0 35	1.66	470
Bone undialyzed	0.725	1.16	160
Same preparation dialyzed 3 wks. at 2°	0.444	1 15	260

like compound by some other cation) and despite electrodialysis for varying lengths of time through cellophane, we have not yet obtained anything approaching a complete inactivation which could be reversed by the addition of Mg. Any preparation which has been inactivated completely in any way will not in our experience regain any of its activity on the addition of Mg.

There seems to be, in fact, some small residue of phosphatase activity in all animal tissue extracts examined which it is not possible to eliminate by dialysis or electrodialysis. It may be that magnesium is a true coenzyme for animal phosphatase in that without Mg no phosphatase activity can be demonstrated,

and this residual activity may therefore be simply a measure of the amount of Mg tenaciously held by the enzyme. That this view is probably erroneous, and that Mg is merely a specific activator and not an essential coenzyme, is strongly suggested by some of our experiments in which we have found that well dialyzed preparations can frequently stand the addition of appreciable amounts of Mg; *i.e.*, amounts up to q Mg in the reaction mixture between 4 and 5, before they begin to show any activation by it.

TABLE II
Effect of Electrodialysis through Cellophane on Activation of Phosphatase by Mg

Source of phosphatase	Activity in phosphatase units per cc. extract		Activity after adding Mg (original activity = 100)
	Without Mg	With Mg (q Mg = 2.5)	
Kidney undialyzed	1.26	1.56	124
Same preparation after dialysis for 1 hr.	0.68	0.94	138
Same preparation after dialysis for 2 hrs.	0.41	0.57	139
Intestine undialyzed	2.94	4.24	144
Same preparation after dialysis for 1 hr.	2.61	4.13	164
Same preparation after dialysis for $3\frac{1}{2}$ hrs.	2.04	3.29	161
Red blood cells, dog, laked.	0.192	0.782	407
Same preparation after dialysis $1\frac{1}{2}$ hrs.	0.156	0.725	468

These quantities of Mg must be considerably greater than any which can be held by the enzyme after dialysis. In other words, extrapolation of the q Mg activity curve does not point to complete inactivation of phosphatase as the Mg concentration diminishes to a vanishingly small amount.

Examples of the effect of magnesium on dialyzed and undialyzed preparations are shown in Tables I and II.

Another effect of prolonged dialysis seems to be to move the optimal q Mg into a region of somewhat higher Mg concentration. An example of this effect is shown in Table III.

Nature of Substrate—Tissue phosphatases seem to be able to effect the hydrolysis of any mono-ester of phosphoric acid. We have examined the effect of varying quantities of Mg on the hydrolysis of a few such esters by phosphatase. Some of the results are shown in Table IV.

In addition to the above substrates, the hydrolysis of sodium α -glycerophosphate and even of sodium pyrophosphate is stimulated by magnesium. An example of the latter effect is shown in Table V, the experiment being conducted in borate buffer at pH

TABLE III
Effect of Prolonged Dialysis on Position of Optimum qMg (Intestinal Phosphatase)

qMg	Hydrolysis for 2 hrs at 37° with	
	Undialyzed enzyme	Dialyzed enzyme
	mg. P	mg. P
>6 (?)	0.212	0.043
5 25	0.214	0.050
4.75	0.215	0.071
4 25	0.220	0.107
3 75	0.262	0.179
3.25	0.297	0.259
2.75	0.330	0.280
2 25	0.355	0.305
1.75	0.510	0.325
1 25	0.412	0.344
0 95	0.375	0.321
0 75	0.336	0.275

The figures in bold-faced type show the position of optimum qMg.

7.6, the optimum pH for the hydrolysis of pyrophosphate by tissue phosphatases (5). It is very probable that there would have been greater stimulus with a higher Mg concentration except for the insolubility of Mg pyrophosphate, which began to come out of solution in the qMg 2.75 tubes and in all tubes containing a higher concentration of Mg, thus diminishing the concentration of the substrate. The optimum qMg of 3.25 indicated by this experiment is almost certainly factitious.

Concentration of Substrate—A few experiments were carried out to determine whether the position of the optimum qMg was

affected by varying the concentration of the substrate, sodium β -glycerophosphate, with *constant* enzyme concentration. There was no marked change in the optimum qMg between concentrations of 1.5 to 12 gm. per liter of the substrate, though the optimum was definitely sharper at the highest substrate concentration. Another experiment with substrate concentration constant and

TABLE IV

Effect of Mg on Hydrolysis of Various Phosphoric Esters by Phosphatase (Dialyzed Intestinal Preparation)

The results are expressed in mg. of P per 0.5 cc. of enzyme.

qMg	Fermentation sodium hexosediphosphate; duplicate hydrolyses		Sodium salt of guanine nucleotide (yeast nucleic acid); duplicate hydrolyses		Sodium glycerophosphate (enzyme-synthesized from ethylene glycol) hydrolysis
4.45	0.204	0.211	0.128	0.127	0.097
3.45	0.253	0.260	0.179	0.182	0.117
2.45	0.280	0.273	0.241	0.242	0.121
1.75					0.116
1.45	0.220	0.235	0.266	0.272	
0.64	0.121	0.121	0.209	0.209	0.094

TABLE V

Activation by Mg of Hydrolysis of Pyrophosphate by Phosphatase

The results are expressed in mg. of P per 0.5 cc. of enzyme.

qMg	Substrate: sodium pyrophosphate; duplicate hydrolyses	
5	0.219	0.217
3.25	0.251	0.253
2.75	0.209	0.210
2.25	0.073	0.073
1.75	0.026	0.027
1.25	0.009	0.009

varying concentrations of enzyme gave a similar result; i.e., no definite change in the optimum qMg with changing enzyme concentration.

Specificity of Mg As Activator

The effect of a large number of ions, basic and acidic, on the activity of the animal phosphatases has been studied, and the

only radical that consistently and markedly stimulates this activity is magnesium. In Table VI will be found a short summary of the results obtained.

TABLE VI
Effect of Dilute Solutions of Various Ions on Phosphatase Activity

Ion or radical	Effect
(a) Metallic	
Mg.....	Consistent and marked stimulation
Ca.	Either none or slight inhibition. Some anomalies (see text)
Sr, Ba, Li, Zn, K.....	Either none or slight inhibition
Be.....	Slight inhibition, but occasional anomalies
Pb, Ag, Ni, Al, Hg, Cu.....	Inhibition
(b) Acidic	
Iodide, iodate, bromide, carbonate, chloride, sulfate, sulfite	None
Citrate, tartrate, oxalate, cyanide, borate, molybdate	Inhibition
Nitrate.....	None or slight acceleration

TABLE VII
Example of Occasional Activation of Phosphatase by Ca

qCa in reaction mixture	Hydrolysis per cc. enzyme per hr.
	<i>mg. P</i>
Very high	0.265
3.7	0.272
2.7	0.270
2.2	0.313
1.7	0.416
1.3	0.529
0.7	0.401

The effect of calcium is not consistently inhibitory. Occasionally, with Ca concentrations in the neighborhood of qCa 4 or less, there has been slightly increased activity and in one or two experiments with intestinal phosphatase the effect of Ca in stimu-

lating phosphatase activity has been as great as that of Mg. The results of one such experiment gave an optimum qCa of 1.3. (See Table VII.)

The repetition of this experiment with a new sample of $CaCl_2$ prepared from Iceland spar gave similar figures, indicating that under certain conditions Ca will act as a stimulus to phosphatase, no less than Mg. This marked effect of Ca has been observed with two samples of intestinal phosphatase only. We have not obtained any similar effect with kidney, bone, or red blood cell phosphatases,¹ all of which are readily stimulated by Mg. This anomalous action of Ca awaits an explanation.

TABLE VIII
Activation of Undialyzed Bone Phosphatase by Mg ($qMg = 3.5$)

Enzyme derived from	Hydrolysis per cc. enzyme; duplicates				Activity after adding Mg (activity without Mg = 100)
	Without Mg		With Mg		
	mg. P	mg. P	mg. P	mg. P	
Rat kidney, normal	0.383	0.381	0.548	0.548	143
“ “ rachitic.	0.288	0.286	0.413	0.417	144
“ intestine, normal	0.435	0.434	0.580	0.592	135
“ “ rachitic.	0.408	0.415	0.506	0.515	124
“ bone, normal	0.359	0.364	0.464	0.476	130
“ “ rachitic	0.401	0.416	0.537	0.533	131

Does Mg Stimulate Bone Phosphatase?

Some years ago one of us (6) brought forward considerable evidence in support of the suggestion that the phosphatases present in bone, kidney, intestine, and other mammalian tissues were identical. It was therefore with much interest that we examined the statement of Hommerberg that Mg does not activate dialyzed bone phosphatase, whereas dialyzed kidney phosphatase is readily activated.

In some preliminary experiments (one of which is shown in Table VIII) we have shown definitely that the addition of $MgCl_2$

¹ Occasionally, with plasma phosphatase and with a graded series of concentrations of calcium, one of the series (in the neighborhood of $qCa = 3$) will show a slight activation above the normal, suggesting that Ca under certain conditions is capable of activating the enzyme.

(qMg of 3.5) will activate undialyzed bone phosphatase of young rats to about the same extent as it will the kidney or the intestinal enzyme.

Having thus demonstrated the effect of Mg as activator for bone phosphatase, we have examined the question as to whether the optimum Mg concentration for activation is the same for bone as for kidney and intestinal phosphatase. At the same time we have tried the effect of dialysis upon Mg activation.

With an undialyzed rat bone preparation we find an optimum between qMg 2.5 and 3.3, with a maximum activation up to 150 (activity without Mg = 100). On dialysis of the same preparation, there is little if any change in the position of the optimum

TABLE IX
Effect of Mg on Activity of Bone Phosphatase

qMg	Hydrolysis per cc enzyme solution; duplicates	
	<i>mg. P</i>	<i>mg. P</i>
Very high	0.178	0.182
2.9	0.318	0.323
2.4	0.342	0.337
1.9	0.295	0.292
1.4	0.290	0.287
0.9	0.267	0.262
0.4	0.222	0.218

qMg but the maximum activation is now up to about 300 (activity without Mg = 100).

Hommerberg used rabbit bone, and it was just possible that there might be some peculiarity in the action of Mg on the bone phosphatase of this animal. We have therefore carried out an experiment with phosphatase from rabbit bone, after dialysis for 10 days against distilled water. The results are given in Table IX. Magnesium therefore activates bone phosphatase just as it does the kidney or the intestinal enzyme, with an optimum qMg in the same region.

Effect of Mg on Red Blood Cell Phosphatase

The erythrocytes of all animals investigated yield, on laking, a phosphatase which is usually more active per unit volume than

that of the plasma of the same animal, and which can be readily activated by addition of small quantities of magnesium. With the red blood cells of certain animals (dogs and rats) the extent of activation is remarkably great, being in some cases as high as 2000 (activity without Mg = 100), and usually at least 1000. With rabbit and beef red blood cells, on the other hand, the activation though definite is not so striking, being usually from 125 to 400 (activity without Mg = 100).

TABLE X
Effect of Mg and Ca on Red Blood Cell Phosphatase

Figures given show hydrolysis in mg. of P per 1 cc. of red blood cells in 48 hours at 38°, pH 7.6, and are corrected for autolysis.

Source of red blood cells	Mg or Ca in reaction mixture	Hydrolysis, duplicates		Activity after addition of Mg (or Ca). Activity before addition = 100
		mg. P	mg. P	
Dog; cells not washed	Very high	0.079	0.074	100
	2.5 (Mg)	1.14	1.16	1500
Rabbit; cells not washed	Very high	0.45	0.46	100
	2.5 (Mg)	0.72	0.74	160
	2.5 (Ca)	0.41	0.41	90
Rabbit; cells washed 5 times with 0.9 per cent NaCl	Very high	0.33	0.33	100
	2.5 (Mg)	0.45	0.45	135
	2.5 (Ca)	0.23	0.23	70
Rat; cells washed 6 times with 0.9 per cent NaCl	Very high	0.23	0.23	100
	2.5 (Mg)	4.14	4.17	1800
	2.5 (Ca)	0.17	0.17	75
Beef; cells washed 4 times with saline	Very high	0.18	0.19	100
	2.5 (Mg)	0.30	0.31	160
	2.5 (Ca)	0.066	0.066	36

Typical findings are given in Table X, showing the striking extent of the activation by Mg, and the generally inhibitory effect of the same molar concentration of Ca.

The very marked activation given by Mg with dog and rat red blood cell phosphatase and the relatively low activation given by Mg with beef and rabbit red blood cells seem to be characteristic for the species. We have obtained repeatedly the same effects with the erythrocyte phosphatase of these animals.

The effect of Mg on rat red blood cells was so striking that a few

experiments were carried out on the time relationships of the enzymic hydrolysis of sodium β -glycerophosphate in the presence and in the absence of Mg. One such experiment is shown in Table XI.

Optimum qMg for Red Blood Cell Phosphatase—This was found to be at about qMg 2.4, which is within the limits of the optimum

TABLE XI
Activation of Red Blood Cell Phosphatase by Mg. Substrate; Sodium β -Glycerophosphate

Time	Hydrolysis in absence of added Mg	Hydrolysis in 0.0033 M MgCl_2 (qMg = 2.5)	Ratio $\frac{\text{Hydrolysis without Mg}}{\text{Hydrolysis with Mg}}$
<i>hrs.</i>	<i>mg. P</i>	<i>mg. P</i>	
3	0.012	0.456	1:38
6	0.018	0.612	1:34
19	0.050	1.74	1:35
48	0.082	3.03	1:37
97	0.099	3.53	1:36

TABLE XII
Optimum qMg for Rat Red Blood Cell Phosphatase, Acting for 48 Hours at 38°, pH 7.6; Chloroform As Antiseptic

qMg in final reaction mixture	Hydrolysis per cc. red blood cells in 48 hrs; duplicates	
	<i>mg. P</i>	<i>mg. P</i>
Very high (4 or more)	0.29	0.29
3.4	1.90	1.90
2.9	3.58	3.52
2.4	4.62	4.49
1.9	3.58	3.50
1.4	3.15	3.21
0.9	2.85	2.90

The figures in bold-faced type show the approximate position of the optimum qMg.

qMg for other animal phosphatases. Results of one typical experiment are shown in Table XII.

It will be observed from Table XII what an extraordinarily delicate test for Mg is this specific activation of rat red blood cell phosphatase. Amounts of Mg of the order of 1 cc. of 0.001 M may readily be detected by this means.

Since the phosphatase activity of red blood cells was so low in absence of added Mg, it was thought that by dialysis after laking or by repeated washing of the cells with isotonic NaCl solution it might be possible to reduce their phosphatase effect to zero; i.e., if all the Mg could be washed away, the cell enzyme would completely lose its activity. However, despite long continued dialysis and repeated washing, it has only been found possible to obtain trifling reductions in the activity in absence of Mg, with little change in the activity when Mg is added. An example of the results of repeated washing of the cells with isotonic NaCl is shown in Table XIII.

TABLE XIII

Effect of Repeated Washing with Isotonic NaCl on Phosphatase Activity of Red Blood Cells

Rat red blood cells, acting for 48 hours at 38°, pH 7.6; chloroform as antiseptic.

No of washings	Hydrolysis without Mg; duplicates		Hydrolysis at $q\text{Mg} = 2.15$; duplicates	
	mg. P	mg. P	mg P	mg. P
0	0.35	0.41	3.15	3.10
1	0.33	0.38	3.93	3.88
2	0.44	0.44	3.48	3.48
3	0.45		3.43	3.48
4	0.39	0.40	4.05	4.33
5	0.35	0.33	3.71	3.82
10	0.29	0.31	3.42	

There is a suggestion from Table XIII that washing removes some substance that inhibits the full extent of Mg activation.

Whether or not Mg is to be considered a coenzyme for animal phosphatases is a matter of definition. If a coenzyme is defined as a specific activator of relatively simple constitution in absence of which an enzyme shows *no* activity, then it has not yet been shown that Mg is a true coenzyme. Thus even after prolonged dialysis or electrodialysis complete inactivation, reversible on addition of Mg, of any phosphatase preparation has never been obtained. Moreover, the activator for animal phosphatase (Mg) markedly increases the rate of hydrolysis but not apparently that of synthesis by this enzyme. (Experiments dealing with the effect of Mg and other ions on synthesis by phosphatase, and on

the position of equilibrium are not yet completed, and will be reported in a later paper.) For the present the rather vaguer term "activator" or "specific activator" will be used to describe the function of Mg in the phosphatase system.

An aspect of Mg activation which must receive attention is the possible mechanism by which this specific activation is brought about. (a) Erdtman (1) has shown that the activator is probably not a protector of the enzyme against autodestruction. This, in any case, would seem to be precluded in particular by the linear character of the hydrolysis either with or without Mg in the early stages of a reaction when relatively small quantities of enzyme are present, and generally by the considerable stability of the dialyzed or the undialyzed enzyme preparation. (b) By demonstrating that a small quantity of activator will assist in the liberation of a very much greater amount of inorganic phosphate, he has also eliminated the possibility that the activation is due to the activator having a much greater affinity than the enzyme for inorganic phosphate, and hence preventing the inactivation of the enzyme by combining with the phosphate as quickly as it is produced. (c) Erdtman suggests that the enzyme alone is more strongly inhibited by phosphate than the enzyme + activator; *i.e.*, that the affinity between the enzyme-activator complex and inorganic phosphate is much lower than that between the enzyme and phosphate. He seems to be too much impressed by the inhibitory effect of the presence of inorganic phosphate on phosphatase activity. (d) We have found, by making determinations at very short intervals after the beginning of hydrolysis, that the effect of Mg is obvious from the start; *i.e.*, before the concentration inorganic phosphate is appreciable. Moreover, *small* quantities of inorganic phosphate, added at the beginning, do not perceptibly inhibit the initial rate of hydrolysis, either in presence or absence of added Mg. The function of Mg in the reaction cannot therefore be entirely the protection of the enzyme against phosphate. We have repeated the experiment that Erdtman recorded as supporting the view quoted in (c) above and find that a precipitate is produced in the reaction mixture under the conditions he described, which probably renders a little uncertain any conclusion drawn from this experiment.

In view of the experiments we have described in this paper and

many others not mentioned here, it seems to us that the Mg activation of phosphatase is unlikely to be an inhibition of an inhibition, but is much more satisfactorily explicable as follows. (e) The colloidal enzyme particle in absence of Mg is either not active, or more probably has only a relatively low phosphatase activity. On addition of small amounts of Mg for which the enzyme has a high adsorptive capacity, the ions of the former are adsorbed by the colloid to form each an enzymically active center. Apart from a small residual activity, the number of unencumbered active centers produced by adsorbed Mg determines the enzymic capacity of the colloid. The number of active centers increases with increase in the amount of Mg in the fluid in which the colloid is dissolved until the maximum number of centers for a given amount of enzyme is reached. (This may possibly be something of the nature of a monomolecular layer of Mg at the colloid-water interface.) Further addition of Mg, which still continues to be adsorbed, now increases the thickness of the Mg layer at this interface and interferes with the access of fresh glycerophosphate or other substrate to the active centers, explaining the rather rapid diminution in rate of hydrolysis as the Mg concentration increases beyond the optimum. We look on the enzyme-Mg complex as having a greater affinity for glycerophosphate, and as showing a greater rate of breakdown of the enzyme-Mg-glycerophosphate complex to enzyme-Mg, glycerol, and phosphate than the enzyme alone, a conclusion borne out by the experiments we have made on enzymic synthesis in the presence and absence of Mg. The foregoing is, of course, little more than a working hypothesis. It does not exclude the possibility that the type of activation suggested by Erdtman may under certain conditions play some part in the system.

We cannot pretend as yet to be able to answer the question, why is the activation so specific? Why does not Ca, Be, Sr, or Ba activate also? In the classical case of the stimulation of salivary amylase by chlorides it is found that bromides, and to a lesser extent, iodides will also activate this enzyme, but in the case of phosphatase, Mg seems to be almost absolutely specific. We have however, with one or two enzyme preparations only, observed reproducible activation by Ca as well as Mg, so that the specificity of Mg cannot be said to be absolute. It seems prob-

able that the activating effect is not due to the peculiar character of any particular chemical compound of Mg (*e.g.* the insolubility in water of the pyrophosphate) but rather to a change in the character of the forces at the enzyme-water interface, usually brought about by Mg, but which may occasionally, when the colloidal micelle is in some unusual condition, be also effected by other activators such as Ca.

SUMMARY

The findings of Erdtman that Mg acts as a specific activator to the hydrolytic action of dialyzed kidney phosphatase on glycerophosphate have been confirmed and extended. It has been shown that Mg will activate all the animal phosphatases which have so far been presented to it, namely those from kidney, intestinal mucosa, bone, liver, red blood cells, and blood plasma, *either before or after dialysis*, though dialysis or electrodialysis increases the percentage activation by Mg.

The increased activity of the bone enzyme in presence of a wide range of Mg concentrations does not agree with the statement of Hommerberg that bone phosphatase differs from kidney phosphatase in that the former is insusceptible to the presence of Mg.

Mg activates the enzymic hydrolysis of α - and β -glycerophosphates, of hexosediphosphate, of guanine nucleotide, and even of pyrophosphate. The optimum concentration of Mg has been determined for several phosphatase preparations. It usually lies between $q\text{Mg}$ 1.7 and 3.3 for different preparations under different conditions ($q\text{Mg}$ = negative logarithm of molarity of Mg in reaction mixture).

Increases of phosphatase activity of over 1800 per cent have been observed on adding Mg to red blood cell preparations from the rat or the dog, but the activation of corresponding preparations from the rabbit or the ox is much less marked.

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CHEMICAL STRUCTURE AND OPTICAL ROTATION

I. THE CONFIGURATIONAL RELATIONSHIP OF DISUBSTITUTED PROPIONIC ACIDS CONTAINING A PHENYL GROUP

II. ON THE OPTICALLY ACTIVE TRISUBSTITUTED METHANES CONTAINING A PHENYL GROUP

By P. A. LEVENE AND R. E. MARKER

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York)

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This paper deals in a general way with the effect on rotation of the phenyl group attached directly to the asymmetric carbon atom. The *first* object of the investigation was the correlation of the disubstituted propionic acids containing a phenyl group; the *second*, the correlation of the above series with the corresponding series of disubstituted acetic acids; the *third*, the correlation of the optically active hydrocarbons of the series of trisubstituted methane containing a phenyl group. The observations on the substances of the last group should serve as a check for the conclusions reached regarding the configurational relationship of the substituted acetic and propionic acids.

A. Correlation of 3-Methylphenyl-, 3-Ethylphenyl-, and 3-Propylphenylpropionic Acids

The configurations of these three substances were correlated by the method employed for the correlation of the configurations of the disubstituted propionic acids previously described.¹ The method of correlation is based on the evident requirement that configurationally related methylphenyl- and ethylphenylpropionic acids should lead to methylethylphenylmethanes of opposite rotations.

¹ Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 77, 687 (1931).

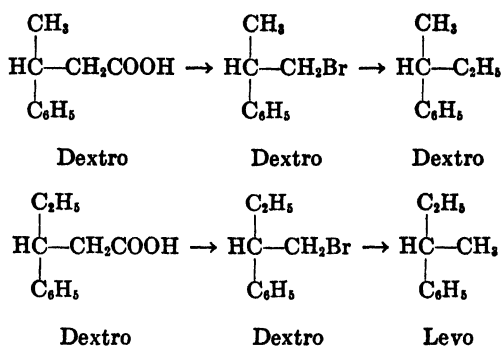


TABLE I

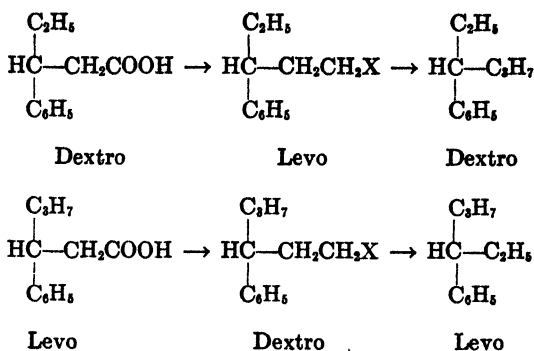
Configurally Related Compounds Derived from Phenylpropionic Acids
 $[\text{M}]_D^{25}$ *

	$-\text{CH}_2\text{COOH}$	$-\text{CH}_2\text{COOC}_2\text{H}_5$	$-\text{CH}_2\text{CH}_2\text{OH}$	$-\text{CH}_2\text{CH}_2\text{Br}$	$-\text{CH}_3$	$-\text{C}_2\text{H}_5$	$-\text{C}_2\text{H}_7$ (n)
$\begin{array}{c} \text{CH}_3 \\ \\ \text{HC}- \\ \\ \text{C}_6\text{H}_5 \end{array}$	+20.31	+14.23	+13.72	+39.45	0	+9.14	
$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{HC}- \\ \\ \text{C}_6\text{H}_5 \end{array}$	+31.64†	+15.27	+7.46	+50.46	-2.59	0	+0.91
$\begin{array}{c} \text{C}_2\text{H}_7 \text{ (n)} \\ \\ \text{HC}- \\ \\ \text{C}_6\text{H}_5 \end{array}$	-11.39	-7.60	-6.47	-26.42		-1.12	0

* All rotations are in homogeneous state unless specified.

† In benzene.

Likewise 3-ethylphenyl- and 3-propylphenylpropionic acids are configurationally related when the ethylpropylphenylmethanes derived from them rotate in opposite directions.

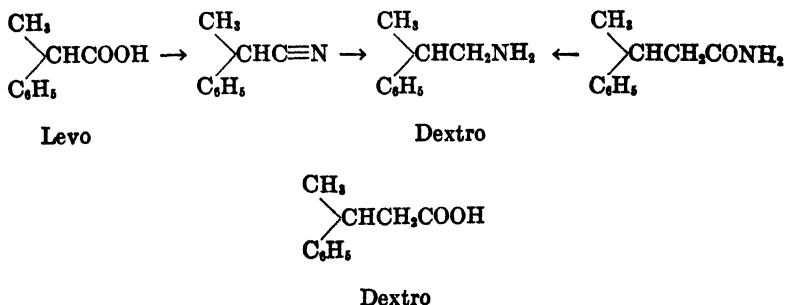


The observations are summarized in Table I.

It can be seen from Table I that *dextro*-methyl and *dextro*-ethylphenyl- and *levo*-propylphenylacetic acids and also that *dextro*-1,3-methylphenyl*dextro*-1,1-ethylphenylpropionic and *levo*-1,1-propylphenylpropionic acids are configurationally related.

B. Correlation of Configurations of Disubstituted Derivatives of Acetic and Propionic Acids

In order to correlate the configurations of 3-methylphenyl and propionic and methylphenylacetic acids, each is converted into 2-methylphenylethylamine (1).



The results obtained by this method are summarized in Table II.

From Table II it can be seen that configurationally related disubstituted acetic and propionic acids rotate in the opposite directions.

TABLE II
Relationship of Phenylacetic Acids to Phenylpropionic Acids
 $[M]_D^{25}$

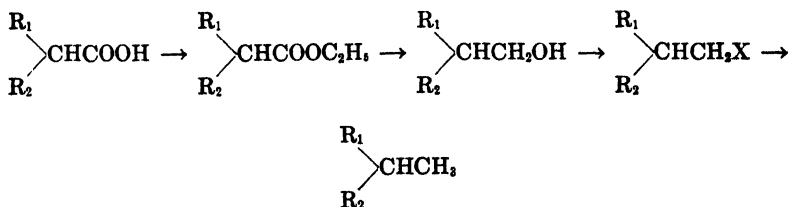
	—COOH	—CH ₂ NH ₂	—CH ₂ COOH*
$\begin{array}{c} \text{CH}_3 \\ \\ \text{HC—} \\ \\ \text{C}_6\text{H}_5 \end{array}$	Levo†	+13.31	+1.56
$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{HC—} \\ \\ \text{C}_6\text{H}_5 \end{array}$	Levo†	—4.86	+4.85
$\begin{array}{c} \text{C}_3\text{H}_7 (n) \\ \\ \text{HC—} \\ \\ \text{C}_6\text{H}_5 \end{array}$	Dextro†	Levo (HBr)	Levo

* See Table I for configurational relationship of these acids.

† Levene, P. A., Mikeska, L. A., and Passoth, K., *J. Biol. Chem.*, **88**, 30 (1930).

Configurational Relationships of Optically Active Hydrocarbons of the Series of Substituted Methanes Containing a Phenyl Group

The correlation of the configurations of the hydrocarbons of this series is based upon the method of their preparation. All hydrocarbons were prepared from the disubstituted acids by the following set of reactions.



The hydrocarbons containing methyl, ethyl, and propyl groups were derived from the acids the configurations of which have been

TABLE III
Configurally Related Phenyl Acids
[M]_D²⁵

	—COOH	—CH ₂ COOH	—CH ₂ CH ₂ CH ₂ COOH
$\begin{array}{c} \text{CH}_3 \\ \\ \text{HC—} \\ \\ \text{C}_6\text{H}_5 \end{array}$	Levo*	+20.31	+11.62
$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{HC—} \\ \\ \text{C}_6\text{H}_5 \end{array}$	Levo*	+31.64	+8.78
$\begin{array}{c} \text{C}_3\text{H}_7 \text{ (n)} \\ \\ \text{HC—} \\ \\ \text{C}_6\text{H}_5 \end{array}$	Dextro*	—11.39	—1.66

* Levene, P. A., Mikeska, L. A., and Passoth, K., *J. Biol. Chem.*, **88**, 30 (1930).

TABLE IV
Configurally Related Esters of Phenyl Acids
[M]_D²⁵

	—COOC ₂ H ₅	—CH ₂ COOC ₂ H ₅	—CH ₂ CH ₂ CH ₂ COOC ₂ H ₅
$\begin{array}{c} \text{CH}_3 \\ \\ \text{HC—} \\ \\ \text{C}_6\text{H}_5 \end{array}$	Levo*	+14.23	+9.21
$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{HC—} \\ \\ \text{C}_6\text{H}_5 \end{array}$	Levo*	+15.27	+4.95
$\begin{array}{c} \text{C}_3\text{H}_7 \text{ (n)} \\ \\ \text{HC—} \\ \\ \text{C}_6\text{H}_5 \end{array}$	Dextro*	—7.60	—1.09

* Levene, P. A., Mikeska, L. A., and Passoth, K., *J. Biol. Chem.*, **88**, 30 (1931).

TABLE V
Configurally Related Phenyl Carbinols
 $[M]_D^{25}$

	$-\text{CH}_2\text{OH}$	$-\text{CH}_2\text{CH}_2\text{OH}$	$-\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$
$\begin{array}{c} \text{CH}_3 \\ \\ \text{HC}- \\ \\ \text{C}_6\text{H}_5 \end{array}$	Levo*	+13.72	+10.57
$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{HC}- \\ \\ \text{C}_6\text{H}_5 \end{array}$	Levo*	+7.46	+4.67
$\begin{array}{c} \text{C}_3\text{H}_7 \text{ (n)} \\ \\ \text{HC}- \\ \\ \text{C}_6\text{H}_5 \end{array}$		-6.47	-2.09

* Levene, P. A., Mikeska, L. A., and Passoth, K., *J. Biol. Chem.*, **88**, 30 (1930).

TABLE VI
Configurally Related Phenyl Halides
 $[M]_D^{25}$

	$-\text{CH}_2\text{Cl}$	$-\text{CH}_2\text{CH}_2\text{Br}$	$-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{Br}$
$\begin{array}{c} \text{CH}_3 \\ \\ \text{HC}- \\ \\ \text{C}_6\text{H}_5 \end{array}$	Dextro*	+39.45	+14.72
$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{HC}- \\ \\ \text{C}_6\text{H}_5 \end{array}$	Levo*	+50.46	+12.56
$\begin{array}{c} \text{C}_3\text{H}_7 \text{ (n)} \\ \\ \text{HC}- \\ \\ \text{C}_6\text{H}_5 \end{array}$		-26.42	-5.39

* Levene, P. A., Mikeska, L. A., and Passoth, K., *J. Biol. Chem.*, **88**, 30 (1930).

discussed in the two preceding sections. The configurations of the hydrocarbons, the highest aliphatic radicle of which was a butyl, could be established by their synthesis from disubstituted 1,1-valeric acids. The latter, indeed, were prepared from the disubstituted propionic acids through the malonic ester synthesis. The intermediate derivatives leading to the lower hydrocarbons

TABLE VII
Experimental Values of Configurationally Related Compounds in Synthesis of Hydrocarbons, Containing a Phenyl Group
[M]_D²⁵

	$-\text{CH}_2\text{CH}_2\text{Br}$	$-\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH}$	$-\text{CH}_2\text{CH}_2\text{CH}_2\text{COOC}_2\text{H}_5$	$-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$	$-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{Br}$	$-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$
$\begin{array}{c} \text{CH}_3 \\ \\ \text{HC}- \\ \\ \text{C}_6\text{H}_5 \end{array}$	+12.84	+3.86	+3.06	+3.51	+4.89	+3.18
$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{HC}- \\ \\ \text{C}_6\text{H}_5 \end{array}$	+25.08	+4.37	+2.46	+2.82	+6.25	+1.71
$\begin{array}{c} \text{C}_3\text{H}_7(n) \\ \\ \text{HC}- \\ \\ \text{C}_6\text{H}_5 \end{array}$	-28.56	-1.80	-1.19	-2.27	-5.86	+1.18

are given in Tables III to VI. Those leading to the hydrocarbons containing a butyl radicle are given in Table VII. The hydrocarbons are tabulated in Table VIII.

The significant feature of Table VIII is the fact that it demonstrates the relationships between the direction of rotation and the allocation of the heavier aliphatic group. A rearrangement be-

TABLE VIII
Configurationaly Related Phenyl Hydrocarbons. $[M]_D^{25}$

$\begin{array}{c} \text{CH}_3 \\ \\ \text{HC}-\text{CH}_2\text{COOH} \\ \\ \text{C}_6\text{H}_5 \end{array}$ + 20.31	$\begin{array}{c} \text{CH}_3 \\ \\ \text{HC}-\text{CH}_3 \\ \\ \text{C}_6\text{H}_5 \end{array}$ 0	$\begin{array}{c} \text{CH}_3 \\ \\ \text{HC}-\text{C}_2\text{H}_5 \\ \\ \text{C}_6\text{H}_5 \end{array}$ + 9.14	$\begin{array}{c} \text{CH}_3 \\ \\ \text{HC}-\text{C}_2\text{H}_7 (n) \\ \\ \text{C}_6\text{H}_5 \end{array}$ + 9.86	$\begin{array}{c} \text{CH}_3 \\ \\ \text{HC}-\text{C}_4\text{H}_9 (n) \\ \\ \text{C}_6\text{H}_5 \end{array}$ + 9.86
$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{HC}-\text{CH}_2\text{COOH} \\ \\ \text{C}_6\text{H}_5 \end{array}$ + 31.64*	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{HC}-\text{CH}_3 \\ \\ \text{C}_6\text{H}_5 \end{array}$ Levo†	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{HC}-\text{C}_2\text{H}_5 \\ \\ \text{C}_6\text{H}_5 \end{array}$ 0	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{HC}-\text{C}_2\text{H}_7 (n) \\ \\ \text{C}_6\text{H}_5 \end{array}$ + 0.91	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{HC}-\text{C}_4\text{H}_9 (n) \\ \\ \text{C}_6\text{H}_5 \end{array}$ + 3.44
$\begin{array}{c} \text{C}_2\text{H}_7 (n) \\ \\ \text{HC}-\text{CH}_2\text{COOH} \\ \\ \text{C}_6\text{H}_5 \end{array}$ - 11.39	$\begin{array}{c} \text{C}_2\text{H}_7 (n) \\ \\ \text{HC}-\text{CH}_3 \\ \\ \text{C}_6\text{H}_5 \end{array}$	$\begin{array}{c} \text{C}_2\text{H}_7 (n) \\ \\ \text{HC}-\text{C}_2\text{H}_5 \\ \\ \text{C}_6\text{H}_5 \end{array}$ - 1.12	$\begin{array}{c} \text{C}_2\text{H}_7 (n) \\ \\ \text{HC}-\text{C}_2\text{H}_7 (n) \\ \\ \text{C}_6\text{H}_5 \end{array}$ 0	$\begin{array}{c} \text{C}_2\text{H}_7 (n) \\ \\ \text{HC}-\text{C}_4\text{H}_9 (n) \\ \\ \text{C}_6\text{H}_5 \end{array}$ + 1.09

* In benzene.

† Relative values not known.

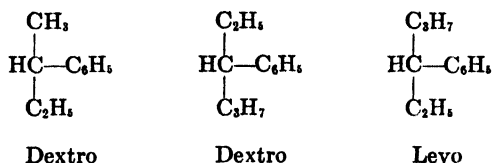
tween the relative positions of the heavier and lighter aliphatic groups leads to a change in rotation. This conduct is to be expected on the basis of the observations on the optically active hydrocarbons of the aliphatic series. Hence, there need be no doubt of the correctness of the relationship of the hydrocarbons as given in Table VIII. This fact in its turn gives confidence in the earlier conclusions as to the configurational relationship of the acids from which they are derived.

Summary of Results

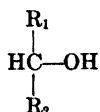
The results of the major part of the observations discussed in the preceding section are summarized in Table IX.

A mere glance at the structure and the rotations of these substances brings out certain general relationships between chemical structure and optical activity.

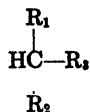
1. The rotations of the hydrocarbons demonstrate the effect of the respective positions of the lighter and heavier aliphatic groups on the direction of the rotation. Thus, presenting the substance in the form



there appears a relationship which has previously been noted in the case of secondary carbinols of the type



and of the aliphatic hydrocarbons of the type



in which the position of the heavier group (R_2) determined the direction of rotation.

TABLE IX
Configurally Related Compounds Containing a Phenyl Group. $[M]_D^{25}$

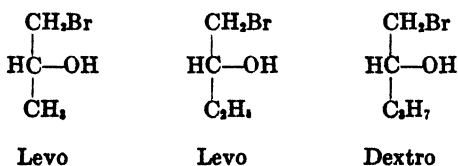
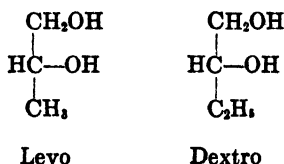
$\begin{array}{c} \text{CH}_3 \\ \\ \text{HC} - \text{C}_6\text{H}_5 \\ \\ \text{CH}_3 \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_2\text{Cl} \\ \\ \text{CH}_2\text{OH} \\ \\ \text{COOC}_2\text{H}_5 \\ \\ \text{COOH} \\ \\ \text{CH}_2\text{NH}_2 \end{array}$	$\begin{array}{c} 0 \\ + 2.5 \\ - 22.1^* \\ \text{Levo} \\ - 96.8 \\ + 23.9 \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{HC} - \text{C}_6\text{H}_5 \\ \\ \text{C}_6\text{H}_5 \end{array}$	$\begin{array}{c} \text{C}_6\text{H}_5 \\ \\ \text{CH}_2\text{CH}_2\text{Br} \\ \\ \text{CH}_2\text{CH}_2\text{OH} \\ \\ \text{CH}_2\text{COOC}_2\text{H}_5 \\ \\ \text{CH}_2\text{COOH} \\ \\ \text{CH}_2\text{NH}_2 \end{array}$	$\begin{array}{c} + 9.14 \\ + 39.45 \\ + 13.72 \\ + 14.23 \\ + 20.31 \\ + 1.56 \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{HC} - \text{C}_6\text{H}_5 \\ \\ \text{C}_6\text{H}_5 \end{array}$	$\begin{array}{c} \text{C}_6\text{H}_5 \\ \\ \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{Br} \\ \\ \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH} \\ \\ \text{CH}_2\text{CH}_2\text{CH}_2\text{COOC}_2\text{H}_5 \\ \\ \text{CH}_2\text{CH}_2\text{CH}_2\text{COOH} \end{array}$	$\begin{array}{c} + 9.86 \\ + 14.72 \\ + 10.57 \\ + 9.21 \\ + 11.62 \end{array}$
$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{HC} - \text{C}_6\text{H}_5 \\ \\ \text{C}_6\text{H}_5 \end{array}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{CH}_2\text{Br} \\ \\ \text{CH}_2\text{OH} \\ \\ \text{COOC}_2\text{H}_5 \\ \\ \text{COOH} \\ \\ \text{CH}_2\text{NH}_2 \end{array}$	$\begin{array}{c} \text{Levo} \\ - 17.8 \\ - 28.3 \\ - 139.3^* \\ - 139.4 \\ - 6.8 \end{array}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{HC} - \text{C}_6\text{H}_5 \\ \\ \text{C}_6\text{H}_5 \end{array}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{CH}_2\text{CH}_2\text{Br} \\ \\ \text{CH}_2\text{CH}_2\text{OH} \\ \\ \text{CH}_2\text{COOC}_2\text{H}_5 \\ \\ \text{CH}_2\text{COOH} \\ \\ \text{CH}_2\text{NH}_2 \end{array}$	$\begin{array}{c} 0 \\ + 50.46 \\ + 7.46 \\ + 15.27 \\ + 31.64 \\ - 4.86 \end{array}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{HC} - \text{C}_6\text{H}_5 \\ \\ \text{C}_6\text{H}_5 \end{array}$	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{Br} \\ \\ \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH} \\ \\ \text{CH}_2\text{CH}_2\text{CH}_2\text{COOC}_2\text{H}_5 \\ \\ \text{CH}_2\text{CH}_2\text{CH}_2\text{COOH} \end{array}$	$\begin{array}{c} + 3.44 \\ + 12.56 \\ + 4.67 \\ + 4.95 \\ + 8.78 \end{array}$
$\begin{array}{c} \text{C}_6\text{H}_5 \\ \\ \text{HC} - \text{C}_6\text{H}_5 \\ \\ \text{C}_6\text{H}_5 \end{array}$	$\begin{array}{c} \text{C}_6\text{H}_5 \\ \\ \text{CH}_2\text{CH}_2\text{Br} \\ \\ \text{CH}_2\text{OH} \\ \\ \text{COOC}_2\text{H}_5 \\ \\ \text{COOH} \\ \\ \text{CH}_2\text{NH}_2 \end{array}$	$\begin{array}{c} + 40.4^* \\ + 27.7^* \\ - 5.2 \end{array}$	$\begin{array}{c} \text{C}_6\text{H}_5 \\ \\ \text{HC} - \text{C}_6\text{H}_5 \\ \\ \text{C}_6\text{H}_5 \end{array}$	$\begin{array}{c} \text{C}_6\text{H}_5 \\ \\ \text{CH}_2\text{CH}_2\text{Br} \\ \\ \text{CH}_2\text{CH}_2\text{OH} \\ \\ \text{CH}_2\text{COOC}_2\text{H}_5 \\ \\ \text{CH}_2\text{COOH} \\ \\ \text{CH}_2\text{NH}_2 \end{array}$	$\begin{array}{c} - 1.12 \\ - 26.42 \\ - 6.47 \\ - 7.60 \\ - 11.39 \\ \text{Levo} \end{array}$	$\begin{array}{c} \text{C}_6\text{H}_5 \\ \\ \text{HC} - \text{C}_6\text{H}_5 \\ \\ \text{C}_6\text{H}_5 \end{array}$	$\begin{array}{c} \text{C}_6\text{H}_5 \\ \\ \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{Br} \\ \\ \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH} \\ \\ \text{CH}_2\text{CH}_2\text{CH}_2\text{COOC}_2\text{H}_5 \\ \\ \text{CH}_2\text{CH}_2\text{CH}_2\text{COOH} \end{array}$	$\begin{array}{c} + 1.18 \\ - 5.39 \\ - 2.09 \\ - 1.09 \\ - 1.66 \end{array}$

In ether

2. The rotations of the configurationally related disubstituted acetic and propionic acids containing a phenyl group clearly show that the effect of a polar group or of a group absorbing in the near ultra-violet region differs with the distance of this group from the asymmetric carbon atom. These observations are in harmony with those of the earlier workers² on relationship between chemical structure and optical activity, as well as with the earlier observations made in this laboratory.¹

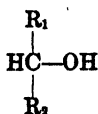
3. The fact that configurationally related ethylphenyl- and propylphenylacetic acids rotate in opposite directions and that configurationally related ethylphenyl- and propylphenylpropionic acids likewise rotate in opposite directions need special consideration.

In a way, similar relationships have been observed in the aliphatic series, in substances containing two polar groups, as can be seen from the following figures.



II

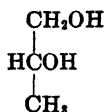
If the rotations of these substances are compared with those of simple secondary carbinols of the type



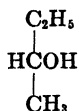
¹ Drude, P., *Optiks*, Leipsic (1900). Tschugaeff, L., *Tr. Faraday Soc.*, 10, 70 (1914). Kuhn, W., *Tr. Faraday Soc.*, 28, 293 (1930).

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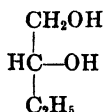
then it will have to be concluded that in



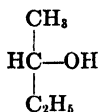
the group $-\text{CH}_2\text{OH}$ functions as the heavier group as in



whereas, in

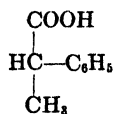


it functions as the lighter group as in

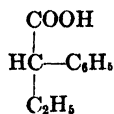


In the case of bromohydrins, the $-\text{CH}_2\text{Br}$ functions as the heavier group in the first two substances. That means it functions as a group heavier than methyl and ethyl but functions as a lighter group compared with propyl and butyl groups.

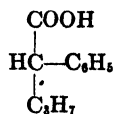
If, then, the substituted acetic and propionic acids are written in an analogous way; namely,



Levo

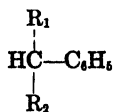


Levo



Dextro

and if these substances are compared with the hydrocarbons of the type



then it will appear that the group COOH functions as a heavier group than $-\text{CH}_3$ and $-\text{C}_2\text{H}_5$ and functions as a lighter group than $-\text{C}_3\text{H}_7$. The same is true in the case of disubstituted propionic and valeric acids; namely, $-\text{CH}_2\text{COOH}$ and $\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH}$ functions as heavier groups than methyl and ethyl and lighter than propyl.

These considerations lead to still another important conclusion; namely, that the direction of rotation in the individual members of this group of substances is not always determined by the group which possesses an ultraviolet absorption band in the neighborhood of the visible spectrum.

EXPERIMENTAL

Levo-2-Phenylbutyric Acid (4)—The inactive acid was prepared from methylphenylbromomethane and ethyl malonate. 340 gm. of inactive acid were dissolved in 6 liters of hot acetone and 1600 gm. of quinine were added. The acetone solution was allowed to stand in a cold room until crystallization took place. After seven crystallizations the salt was decomposed by shaking with 10 per cent hydrochloric acid. The organic acid was extracted with ether, then fractionated. B.p., 134° at 4 mm.; yield, 55 gm.; $n_D^{25} = 1.5169$; $D_{\frac{25}{4}} = 1.066$.

$$[\alpha]_D^{25} = \frac{-13.20^\circ}{1 \times 1.066} = -12.38^\circ. \quad [M]_D^{25} = -20.31^\circ \text{ (homogeneous)}$$

$$[\alpha]_D^{25} = \frac{-1.25^\circ \times 100}{1 \times 0.5520 \times 20} = -11.32^\circ. \quad [M]_D^{25} = -18.57^\circ \text{ (in benzene)}$$

4.550 mg. substance: 12.180 mg. CO_2 and 3.090 mg. H_2O .

$\text{C}_{10}\text{H}_{12}\text{O}_2$. Calculated. C 73.17, H 7.37

Found. " 72.82, " 7.60

Levo-Ethyl Ester of 2-Phenylbutyric Acid (4)—110 gm. of 2-phenylbutyric acid (4), $[\alpha]_D^{25} = -12.39^\circ$, were mixed with 250 cc. of absolute alcohol and 6 cc. of concentrated sulfuric acid. The mixture was heated for 1 hour on a steam bath. The excess alcohol was distilled off under reduced pressure and the residue extracted with ether. The ether solution was washed with water,

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dried with sodium sulfate, then fractionated. B.p., 111° at 4 mm.; yield, 116 gm.; $n_D^{25} = 1.4918$; $D_4^{25} = 0.996$.

$$[\alpha]_D^{25} = \frac{-7.38^{\circ}}{1 \times 0.996} = -7.41^{\circ}. \quad [M]_D^{25} = -14.24^{\circ} \text{ (homogeneous)}$$

3.540 mg. substance: 9.745 mg. CO_2 and 2.620 mg. H_2O .

$\text{C}_{12}\text{H}_{16}\text{O}_2$. Calculated. C 74.95, H 8.39

Found. " 75.06, " 8.28

Levo-3-Phenyl-1-Butanol—100 gm. of ethyl ester of 2-phenylbutyric acid (4), $[\alpha]_D^{25} = -7.41^{\circ}$, were dissolved in 500 cc. of absolute alcohol and this solution dropped into a suspension of 150 gm. of sodium in 1 liter of boiling toluene with rapid stirring. Alcohol was then added to destroy the excess sodium. The reaction mixture was cooled. Ice was added and the carbinol extracted from the alkaline solution with ether. The ether solution was dried, then fractionated. B.p., 117° at 8 mm.; yield, 76 gm.; $n_D^{25} = 1.5201$; $D_4^{25} = 0.986$.

$$[\alpha]_D^{25} = \frac{-9.02^{\circ}}{1 \times 0.986} = -9.15^{\circ}. \quad [M]_D^{25} = -13.74^{\circ} \text{ (homogeneous)}$$

4.245 mg. substance: 12.465 mg. CO_2 and 3.600 mg. H_2O .

$\text{C}_{10}\text{H}_{14}\text{O}$. Calculated. C 79.94, H 9.40

Found. " 80.07, " 9.49

Levo-1-Bromo-3-Phenylbutane—12 gm. of 3-phenyl-1-butanol, $[\alpha]_D^{25} = -9.15^{\circ}$, were cooled in ice and 15 gm. of phosphorus tribromide slowly added. The product was heated 1 hour on a steam bath, then cooled, and poured on ice. The oily layer was separated, shaken with strong hydrobromic acid solution, extracted with ether, dried, and then fractionated. B.p., 120° at 17 mm.; yield, 15 gm.; $n_D^{25} = 1.5371$; $D_4^{25} = 1.314$.

$$[\alpha]_D^{25} = \frac{-24.33^{\circ}}{1 \times 1.314} = -18.52^{\circ}. \quad [M]_D^{25} = -39.44^{\circ} \text{ (homogeneous)}$$

4.440 mg. substance: 9.190 mg. CO_2 and 2.395 mg. H_2O .

$\text{C}_{10}\text{H}_{12}\text{Br}$. Calculated. C 56.33, H 6.15

Found. " 56.44, " 6.03

Levo-Methylethylphenylmethane—A Grignard reagent was prepared from 6 gm. of magnesium in dry ether and 50 gm. of 1-

bromo-3-phenylbutane, $[\alpha]_D^{25} = -18.52^\circ$. The Grignard reagent was poured on ice and dilute hydrochloric acid and the hydrocarbon extracted with ether. The ether was evaporated and the residue shaken with a small amount of ice-cold concentrated sulfuric acid, washed with sodium carbonate solution, then water, and dried over dry sodium sulfate. It was then distilled from a small piece of metallic sodium. B.p., 68° at 20 mm.; yield, 21 gm.; $D_4^{24} = 0.868$.

$$[\alpha]_D^{24} = \frac{-5.92^\circ}{1 \times 0.868} = -6.82^\circ. \quad [M]_D^{24} = -9.14^\circ \text{ (homogeneous)}$$

4.693 mg. substance: 15.345 mg. CO_2 and 4.445 mg. H_2O .

$\text{C}_{10}\text{H}_{14}$. Calculated. C 89.48, H 10.52

Found. " 89.16, " 10.59

Levo-3-Phenylvaleric Acid (5)—The inactive acid was prepared from ethylphenylbromomethane and ethyl malonate. 356 gm. of the inactive acid were dissolved in 4 liters of hot acetone and 756 gm. of quinine added. The salt was crystallized by letting the solution stand overnight in a cold room. After eight crystallizations it was decomposed by 10 per cent hydrochloric acid and the organic acid recovered as described for 3-methylphenylpropionic acid. B.p., 140° at 4 mm.; yield, 53 gm. This material solidified at room temperature.

$$[\alpha]_D^{25} = \frac{-1.60^\circ \times 100}{1 \times 20 \times 0.4851} = -16.5^\circ. \quad [M]_D^{25} = -29.4^\circ \text{ (in benzene)}$$

4.235 mg. substance: 11.570 mg. CO_2 and 3.015 mg. H_2O .

$\text{C}_{11}\text{H}_{14}\text{O}_2$. Calculated. C 74.11, H 7.92

Found. " 74.50, " 7.99

Levo-Ethyl Ester of 3-Phenylvaleric Acid (5)—100 gm. of 3-phenylvaleric acid (5), $[\alpha]_D^{25} = -16.48^\circ$ (in benzene), were mixed with 250 cc. of absolute alcohol and 6 cc. of concentrated sulfuric acid. The esterification was carried out as described for ethyl ester of 3-methylphenylpropionic acid. B.p., 110° at 2 mm.; yield, 105 gm.

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$$[\alpha]_D^{25} = \frac{-7.30^\circ}{1 \times 0.985} = -7.41^\circ. \quad [M]_D^{25} = -15.02^\circ \text{ (homogeneous)}$$

3.780 mg. substance: 10.480 mg. CO₂ and 3.075 mg. H₂O.
 C₁₃H₁₈O₂. Calculated. C 75.68, H 8.80
 Found. " 75.60, " 9.10

Levo-3-Phenyl-1-Pentanol—100 gm. of the ethyl ester of 3-phenylvaleric acid (5), $[\alpha]_D^{25} = -7.41^\circ$, were dissolved in 500 cc. of absolute alcohol. This solution was dropped into 140 gm. of finely divided sodium in 800 cc. of boiling toluene with stirring. The reduction was carried out as described for 3-phenyl-1-butanol. B.p., 108° at 1 mm.; yield, 65 gm.; $n_D^{25} = 1.5145$; $D_4^{25} = 0.967$.

$$[\alpha]_D^{25} = \frac{-4.38^\circ}{1 \times 0.967} = -4.53^\circ. \quad [M]_D^{25} = -7.43^\circ \text{ (homogeneous)}$$

4.050 mg. substance: 11.995 mg. CO₂ and 3.610 mg. H₂O.
 C₁₁H₁₆O. Calculated. C 80.42, H 9.82
 Found. " 80.76, " 9.97

Levo-1-Bromo-3-Phenylpentane—10 gm. of 3-phenyl-1-pentanol, $[\alpha]_D^{25} = -4.54^\circ$, were brominated by 15 gm. of phosphorus tri-bromide as described for 1-bromo-3-phenylbutane. B.p., 127° at 15 mm.; yield, 12 gm.; $n_D^{25} = 1.5287$; $D_4^{25} = 1.224$.

$$[\alpha]_D^{25} = \frac{-27.21^\circ}{1 \times 1.224} = -22.23^\circ. \quad [M]_D^{25} = -50.47^\circ \text{ (homogeneous)}$$

4.335 mg. substance: 9.225 mg. CO₂ and 2.620 mg. H₂O.
 C₁₁H₁₅Br. Calculated. C 58.17, H 6.66
 Found. " 58.03, " 6.76

Dextro-Ethyl-n-Propylphenylmethane—A Grignard reagent was prepared from 28 gm. of 1-bromo-3-phenylpentane, $[\alpha]_D^{25} = +16.53^\circ$, and 4 gm. of magnesium in dry ether. To this were added 5 gm. of paraformaldehyde; and the mixture was allowed to stand overnight. The Grignard reagent was decomposed in the usual way and the crude carbinol distilled. B.p., 115° at 3 to 4 mm.; yield, 13 gm. This was not purified further.

13 gm. of the carbinol were treated with 25 gm. of phosphorus tribromide as described for 1-bromo-3-phenylbutane. Yield, 12 gm.; b.p., 110° at 3 to 4 mm. A Grignard reagent was prepared from this halide and 1.5 gm. of finely divided magnesium in ether.

This was poured on ice and the hydrocarbon extracted and purified as described for methylethylphenylmethane. B.p., 103° at 25 mm.; yield, 3 gm.; $D_4^{24} = 0.863$.

$$[\alpha]_D^{25} = \frac{+0.49^{\circ}}{1 \times 0.863} = +0.57^{\circ}. \quad [M]_D^{25} = +0.92^{\circ} \text{ (homogeneous)}$$

4.331 mg. substance: 13.994 mg. CO_2 and 4.250 mg. H_2O .

$\text{C}_{12}\text{H}_{18}$. Calculated. C 88.81, H 11.19

Found. " 88.11, " 10.98

Dextro-4-Phenylcaproic Acid (6)—The inactive acid was prepared from *n*-propylphenylbromomethane and ethyl malonate.

460 gm. of the inactive acid were dissolved in 6 liters of boiling acetone and 870 gm. of quinine were added. The solution was allowed to stand in the cold room overnight for crystallization. After six crystallizations the quinine salt was decomposed and the acid extracted as described for 3-methylphenylpropionic acid. B.p., 152° at 4 mm.; yield, 70 gm.; $n_D^{25} = 1.5078$; $D_4^{25} = 1.025$.

$$[\alpha]_D^{25} = \frac{+6.08^{\circ}}{1 \times 1.025} = +5.93^{\circ}. \quad [M]_D^{25} = +11.39^{\circ} \text{ (homogeneous)}$$

4.395 mg. substance: 12.145 mg. CO_2 and 3.305 mg. H_2O .

$\text{C}_{12}\text{H}_{16}\text{O}_2$. Calculated. C 74.95, H 8.39

Found. " 75.35, " 8.41

Dextro-Ethyl Ester of 4-Phenylcaproic Acid (6)—75 gm. of 4-phenylcaproic acid (6), $[\alpha]_D^{25} = +5.93^{\circ}$, were mixed with 175 cc. of absolute alcohol and 4 cc. of sulfuric acid. Esterification was carried out as described for the ethyl ester of 3-methylphenylpropionic acid. B.p., 123° at 2 mm.; yield, 80 gm.; $n_D^{25} = 1.4870$; $D_4^{25} = 0.969$.

$$[\alpha]_D^{25} = \frac{+3.34^{\circ}}{1 \times 0.969} = +3.45^{\circ}. \quad [M]_D^{25} = +7.60^{\circ} \text{ (homogeneous)}$$

3.955 mg. substance: 11.060 mg. CO_2 and 3.360 mg. H_2O .

$\text{C}_{14}\text{H}_{20}\text{O}_2$. Calculated. C 76.31, H 9.16

Found. " 76.25, " 9.50

Dextro-3-Phenyl-1-Hexanol—75 gm. of ethyl ester of 4-phenylcaproic acid (6), $[\alpha]_D^{25} = +3.45^{\circ}$, were dissolved in 300 cc. of

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absolute alcohol and this solution dropped into a suspension of 90 gm. of sodium in 450 cc. of boiling toluene with stirring. The carbinol was isolated as described for 3-phenylbutanol. B.p., 127° at 5 mm.; yield, 55 gm.; $n_D^{25} = 1.5101$; $D_4^{25} = 0.955$.

$$[\alpha]_D^{25} = \frac{+ 2.40^\circ}{1 \times 0.955} = + 2.51^\circ. \quad [M]_D^{25} = + 4.47^\circ \text{ (homogeneous)}$$

3.290 mg. substance: 9.760 mg. CO₂ and 3.030 mg. H₂O.

C₁₃H₁₈O. Calculated. C 80.83, H 10.18

Found. " 80.89, " 10.30

Dextro-1-Bromo-3-Phenylhexane—15 gm. of 3-phenyl-1-hexanol, $[\alpha]_D^{25} = +2.51^\circ$, were brominated by means of 25 gm. of phosphorus tribromide as described for 1-bromo-3-phenylbutane. B.p., 112° at 2 mm.; yield, 16 gm.; $n_D^{25} = 1.5250$; $D_4^{25} = 1.199$.

$$[\alpha]_D^{25} = \frac{+ 13.14^\circ}{1 \times 1.199} = + 10.96^\circ. \quad [M]_D^{25} = + 26.42^\circ \text{ (homogeneous)}$$

4.600 mg. substance: 10.080 mg. CO₂ and 2.935 mg. H₂O.

C₁₃H₁₇Br. Calculated. C 59.73, H 7.11

Found. " 59.74, " 7.14

Dextro-Ethyl-n-Propylphenylmethane—A Grignard reagent was prepared from 4 gm. of magnesium in dry ether and 30 gm. of 1-bromo-3-phenylhexane, $[\alpha]_D^{25} = +10.96^\circ$. The Grignard reagent was poured on ice and the hydrocarbon extracted and purified as described for methylethylphenylmethane. B.p., 105° at 27 mm.; yield, 19 gm.; $D_4^{24} = 0.863$.

$$[\alpha]_D^{24} = \frac{+ 0.58^\circ}{1 \times 0.863} = + 0.67^\circ. \quad [M]_D^{24} = + 1.09^\circ \text{ (homogeneous)}$$

3.605 mg. substance: 11.710 mg. CO₂ and 3.515 mg. H₂O.

C₁₃H₁₈. Calculated. C 88.81, H 11.19

Found. " 88.58, " 10.91

The preparation of this hydrocarbon was repeated starting with 24 gm. of 1-bromo-3-phenylhexane, $[\alpha]_D^{25} = +8.52^\circ$ (homogeneous). A hydrocarbon was obtained which had the following rotation.

$$[\alpha]_D^{24} = \frac{+ 0.48^\circ}{1 \times 0.861} = + 0.56^\circ. \quad [M]_D^{24} = + 0.91^\circ \text{ (homogeneous)}$$

4.331 mg. substance: 14.174 mg. CO₂ and 4.198 mg. H₂O.

C₁₃H₁₈. Calculated. C 88.81, H 11.19

Found. " 89.24, " 10.84

Dextro-2-Phenylbutyric Acid (4)—39 gm. of 2,2-methylphenylethylamine, $[\alpha]_D^{24} = \frac{+9.25^\circ}{1 \times 0.938} = +9.86^\circ$, $[M]_D^{24} = +13.31^\circ$, were slowly dropped into an ether solution of nitrosyl chloride at -50° . The chloride was distilled under reduced pressure and then converted into a Grignard reagent. Carbon dioxide was passed into this reagent for about 15 minutes. The Grignard reagent was decomposed by pouring on ice and hydrochloric acid and extracting with ether. The ether solution was shaken with dilute potassium hydroxide, then separated and the alkaline solution extracted with ether. The solution was acidified, the organic acid extracted with ether, and distilled. B.p., 135° at 4 mm.; yield, 3.5 gm.

$$[\alpha]_D^{25} = \frac{+1.02^\circ}{1 \times 1.066} = +0.96^\circ. \quad [M]_D^{25} = +1.57^\circ \text{ (homogeneous)}$$

3.875 mg. substance: 10.340 mg. CO_2 and 2.568 mg. H_2O .

$\text{C}_{10}\text{H}_{12}\text{O}_2$. Calculated. C 73.13, H 7.37

Found. " 72.76, " 7.41

Dextro-3-Phenylvaleric Acid (5)—57 gm. of 2,2-ethylphenylethylamine, $[\alpha]_D^{24} = -3.26^\circ$, were chlorinated by means of nitrosyl chloride. Yield, 34 gm. A Grignard reagent was prepared from this chloride and magnesium in dry ether. The Grignard reagent was divided into two portions. The first portion was cooled in ice and dry carbon dioxide passed in for 15 minutes. The acid was isolated and purified as described for 2-phenylbutyric acid (4). B.p., 142° at 5 mm.; yield, 3 gm. (solid).

$$[\alpha]_D^{25} = \frac{+0.31^\circ \times 100}{1 \times 0.542 \times 20} = +2.86^\circ. \quad [M]_D^{25} = +5.10^\circ \text{ (in benzene)}$$

5.671 mg. substance: 15.425 mg. CO_2 and 3.945 mg. H_2O .

$\text{C}_{11}\text{H}_{14}\text{O}_2$. Calculated. C 74.11, H 7.92

Found. " 74.17, " 7.79

Levo-Methylethylphenylmethane—The second portion of the Grignard reagent prepared above was poured on ice and hydrochloric acid. The hydrocarbon was isolated and purified as described for the first preparation of methylethylphenylmethane. B.p., 68° at 20 mm.; yield, 3 gm.

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$$[\alpha]_D^{25} = \frac{-1.67^\circ}{1 \times 0.868} = -1.92^\circ. \quad [M]_D^{25} = -2.58^\circ \text{ (homogeneous)}$$

3.994 mg. substance: 13.080 mg. CO₂ and 3.770 mg. H₂O.

C₁₀H₁₄. Calculated. C 89.48, H 10.52

Found. " 89.30, " 10.56

Dextro-3-Phenylheptanoic Acid (7)—35 gm. of sodium were dissolved in 400 cc. of absolute alcohol and 240 gm. of ethyl malonate were added. To this were added 230 gm. of 1-bromo-3-phenylpentane, $[\alpha]_D^{25} = +11.05^\circ$, $[M]_D^{25} = +25.08^\circ$. The product was refluxed for 2 hours, and then the ester was isolated and hydrolyzed by boiling with 3 mols of potassium hydroxide dissolved in 80 per cent alcohol. The alcohol was evaporated and the sodium salt of the malonic acid was acidified with dilute sulfuric acid. The organic acid was extracted with ether, then the ether was evaporated. The acid was heated in a metal bath at 200° until carbon dioxide ceased coming off. It was then distilled. B.p., 162° at 1 mm.; yield, 152 gm.; $D_{44}^{25} = 1.012$.

$$[\alpha]_D^{25} = \frac{+2.15^\circ}{1 \times 1.012} = +2.12^\circ. \quad [M]_D^{25} = +4.31^\circ \text{ (homogeneous)}$$

4.259 mg. substance: 11.720 mg. CO₂ and 3.085 mg. H₂O.

C₁₃H₁₈O₂. Calculated. C 75.67, H 8.80

Found. " 75.04, " 8.10

Dextro-Ethyl Ester of 3-Phenylheptanoic Acid (7)—152 gm. of 3-phenylheptanoic acid(7), $[\alpha]_D^{25} = +2.12^\circ$, $[M]_D^{25} = +4.37^\circ$, were dissolved in 400 cc. of absolute alcohol and 5 cc. of concentrated sulfuric acid were added. The product was heated 1 hour on a steam bath. The excess alcohol was distilled off and the residue washed with water, extracted with ether, and then distilled. B.p., 147° at 1 mm.; yield, 145 gm.; $D_{44}^{25} = 0.969$.

$$[\alpha]_D^{25} = \frac{+1.02^\circ}{1 \times 0.969} = +1.05^\circ. \quad [M]_D^{25} = +2.46^\circ \text{ (homogeneous)}$$

3.735 mg. substance: 10.595 mg. CO₂ and 3.045 mg. H₂O.

C₁₅H₂₂O₂. Calculated. C 76.86, H 9.47

Found. " 77.35, " 9.12

Dextro-3-Phenylheptanol (7)—145 gm. of ethyl ester of 3-phenylheptanoic acid(7), $[\alpha]_D^{25} = +1.05^\circ$, $[M]_D^{25} = +2.46^\circ$, were

dissolved in 500 cc. of absolute alcohol. This was slowly dropped into a suspension of 200 gm. of finely divided sodium in 1 liter of toluene, with vigorous stirring during addition. The excess sodium was destroyed by absolute alcohol, then water was added. The carbinol was extracted and then distilled. B.p., 145° at 1 mm.; yield, 110 gm.; $D_4^{25} = 0.952$.

$$[\alpha]_D^{25} = \frac{+1.40^{\circ}}{1 \times 0.952} = +1.47^{\circ}. \quad [M]_D^{25} = +2.82^{\circ} \text{ (homogeneous)}$$

4.309 mg. substance: 12.860 mg. CO_2 and 3.945 mg. H_2O .

$\text{C}_{13}\text{H}_{20}\text{O}$. Calculated. C 81.18, H 10.49

Found. " 81.39, " 10.24

Dextro-3-Phenyl-7-Bromoheptane—100 gm. of 3-phenylheptanol(7), $[\alpha]_D^{25} = +1.47^{\circ}$, $[M]_D^{25} = +2.82^{\circ}$, were cooled in ice and 200 gm. of phosphorus tribromide were slowly added. The product was heated for 30 minutes on a steam bath, then poured into a flask containing ice. The halide was extracted with ether, then distilled. B.p., 131° at 1 mm.; yield, 103 gm.; $D_4^{25} = 1.176$.

$$[\alpha]_D^{25} = \frac{+2.88^{\circ}}{1 \times 1.176} = +2.45^{\circ}. \quad [M]_D^{25} = +6.25^{\circ} \text{ (homogeneous)}$$

3.805 mg. substance: 8.490 mg. CO_2 and 2.440 mg. H_2O .

$\text{C}_{13}\text{H}_{19}\text{Br}$. Calculated. C 61.15, H 7.51

Found. " 60.84, " 7.17

Dextro-3-Phenylheptane—A Grignard reagent was prepared from 10 gm. of magnesium in 200 cc. of dry ether and 103 gm. of 3-phenyl-7-bromoheptane, $[\alpha]_D^{25} = +2.45^{\circ}$, $[M]_D^{25} = +6.25^{\circ}$. This was poured on ice and after dissolving the magnesium hydroxide with dilute hydrochloric acid, the hydrocarbon was extracted with ether. It was purified as previously described, then distilled. B.p., 105° at 16 mm.; yield, 24 gm.; $D_4^{25} = 0.856$.

$$[\alpha]_D^{25} = \frac{+0.83^{\circ}}{1 \times 0.856} = +0.97^{\circ}. \quad [M]_D^{25} = +1.71^{\circ} \text{ (homogeneous)}$$

2.808 mg. substance: 9.108 mg. CO_2 and 2.915 mg. H_2O .

$\text{C}_{13}\text{H}_{20}$. Calculated. C 88.55, H 11.44

Found. " 88.45, " 11.61

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Levo-4-Phenyloctanoic Acid (8)—13 gm. of sodium were dissolved in 150 cc. of absolute alcohol and 80 gm. of ethyl malonate were added. To this were added 125 gm. of 1-bromo-3-phenylhexane, $[\alpha]_D^{25} = -11.85^\circ$. The product was refluxed 2 hours, then the ester was hydrolyzed by boiling with 150 gm. of potassium hydroxide dissolved in 1 liter of 80 per cent alcohol. The alcohol was evaporated, and the solution acidified with dilute sulfuric acid. The malonic acid was extracted with ether, and after evaporation of the ether was heated in a metal bath at 190° until carbon dioxide ceased coming off. It was then distilled. B.p., 170° at 1 mm.; yield, 85 gm.; $D_4^{25} = 0.999$.

$$[\alpha]_D^{25} = \frac{-0.82^\circ}{1 \times 0.999} = -0.82^\circ. \quad [M]_D^{25} = -1.80^\circ \text{ (homogeneous)}$$

4.700 mg. substance: 13.185 mg. CO_2 and 3.865 mg. H_2O .

$\text{C}_{14}\text{H}_{20}\text{O}_2$. Calculated. C 76.31, H 9.16

Found. " 76.50, " 9.20

Levo-Ethyl Ester of 4-Phenyloctanoic Acid (8)—85 gm. of 4-phenyloctanoic acid (8), $[\alpha]_D^{25} = -0.82^\circ$, were dissolved in 200 cc. of absolute alcohol and 8 cc. of concentrated sulfuric acid were added. The product was refluxed $\frac{1}{2}$ hour, then the excess alcohol distilled, and the ester extracted with ether. It was then distilled. B.p., 152° at 1 mm.; yield, 93 gm.; $D_4^{25} = 0.958$.

$$[\alpha]_D^{25} = \frac{-0.47^\circ}{1 \times 0.958} = -0.49^\circ. \quad [M]_D^{25} = -1.22^\circ \text{ (homogeneous)}$$

3.069 mg. substance: 8.633 mg. CO_2 and 2.720 mg. H_2O .

$\text{C}_{16}\text{H}_{24}\text{O}_2$. Calculated. C 77.36, H 9.75

Found. " 76.70, " 9.91

Levo-4-Phenyloctanol (8)—63 gm. of the ethyl ester of 4-phenyloctanoic acid (8), $[\alpha]_D^{25} = -0.48^\circ$, were dissolved in 300 cc. of absolute alcohol and reduced by dropping into a suspension of 135 gm. of sodium in 675 cc. of toluene with vigorous stirring. Water was added after the sodium was dissolved. The carbinol was extracted with ether, then distilled. B.p., 147° at 1 mm.; yield, 47 gm.; $D_4^{25} = 0.941$.

$$[\alpha]_D^{25} = \frac{-1.04^\circ}{1 \times 0.941} = -1.10^\circ. \quad [M]_D^{25} = -2.28^\circ \text{ (homogeneous)}$$

3.876 mg. substance: 11.575 mg. CO₂ and 3.730 mg. H₂O.

C₁₄H₂₂O. Calculated. C 81.48, H 10.76

Found. " 81.43, " 10.76

Levo-4-Phenyl-8-Bromooctane—47 gm. of 4-phenyloctanol(8), $[\alpha]_D^{25} = -1.10^\circ$, were cooled in ice and 100 gm. of phosphorus tri-bromide were added. The product was heated for 15 minutes on a steam bath. It was then poured on ice and the halide extracted with ether and distilled. B.p., 146° at 1 mm.; yield, 43 gm.; $D_4^{25} = 1.109$.

$$[\alpha]_D^{25} = \frac{-2.42^\circ}{1 \times 1.109} = -2.18^\circ. \quad [M]_D^{25} = -5.87^\circ \text{ (homogeneous)}$$

3.915 mg. substance: 9.020 mg. CO₂ and 2.805 mg. H₂O.

C₁₄H₂₁Br. Calculated. C 62.43, H 7.87

Found. " 62.82, " 8.01

Dextro-4-Phenyloctane—A Grignard reagent was prepared from 42 gm. of 4-phenyl-8-bromooctane, $[\alpha]_D^{25} = -2.18^\circ$, and 5 gm. of magnesium in 100 cc. of ether. This was poured into a flask containing ice and dilute hydrochloric acid. The hydrocarbon was extracted with ether, dried, and distilled. It was purified as previously described, then distilled from sodium. B.p., 119° at 18 mm.; yield, 23 gm.; $D_4^{25} = 0.855$.

$$[\alpha]_D^{25} = \frac{+1.06^\circ}{2 \times 0.855} = +0.62^\circ. \quad [M]_D^{25} = +1.18^\circ \text{ (homogeneous)}$$

2.781 mg. substance: 8.995 mg. CO₂ and 2.920 mg. H₂O.

C₁₄H₂₂. Calculated. C 88.34, H 11.66

Found. " 88.20, " 11.74

Dextro-2-Phenylcaproic Acid (6)—35 gm. of sodium were dissolved in 400 cc. of absolute alcohol and 240 gm. of ethyl malonate were added. To this were added 213 gm. of 1-bromo-3-phenylbutane, $[\alpha]_D^{25} = +6.03^\circ$, $[M]_D^{25} = +12.84^\circ$. The product was refluxed 2 hours on a water bath. The ester was then isolated and hydrolyzed by boiling with 3 mols of potassium hydroxide in 80 per cent alcohol. The excess alcohol was evaporated and the

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potassium salt of the malonic acid was acidified with 25 per cent sulfuric acid. This was extracted with ether and after evaporation of the ether was heated at 190° in a metal bath until CO₂ ceased coming off. The acid was then distilled. B.p., 156° at 1 mm.; yield, 138 gm.; $D_4^{25} = 1.022$.

$$[\alpha]_D^{25} = \frac{+ 2.05^\circ}{1 \times 1.022} = + 2.01^\circ. \quad [M]_D^{25} = + 3.86^\circ \text{ (homogeneous)}$$

5.041 mg. substance: 13.696 mg. CO₂ and 3.718 mg. H₂O.

C₁₃H₁₆O₃. Calculated. C 74.95, H 8.39

Found. " 74.09, " 8.25

Dextro-Ethyl Ester of 2-Phenylcaproic Acid (6)—135 gm. of 2-phenylcaproic acid (6), $[\alpha]_D^{25} = +2.01^\circ$, $[M]_D^{25} = +3.86^\circ$, were dissolved in 300 cc. of absolute alcohol and 8 cc. of concentrated sulfuric acid were added. The product was refluxed on a steam bath for 30 minutes, the excess alcohol distilled off, and the ester extracted with ether. The ether was washed with dilute sodium carbonate solution, then the product distilled. B.p., 145° at 1 mm.; yield, 132 gm.; $D_4^{25} = 0.974$.

$$[\alpha]_D^{25} = \frac{+ 1.36^\circ}{1 \times 0.974} = + 1.40^\circ. \quad [M]_D^{25} = + 3.08^\circ \text{ (homogeneous)}$$

2.795 mg. substance: 7.814 mg. CO₂ and 2.390 mg. H₂O.

C₁₄H₁₈O₃. Calculated. C 76.31, H 9.16

Found. " 76.23, " 9.56

Dextro-2-Phenylhexanol (6)—122 gm. of ethyl ester of 2-phenylcaproic acid (6), $[\alpha]_D^{25} = +2.01^\circ$, $[M]_D^{25} = +3.86^\circ$, were dissolved in 400 cc. of absolute alcohol and reduced (in 40 gm. quantities) by slowly dropping into a suspension of 180 gm. of sodium in 900 cc. of boiling toluene with vigorous stirring. Water was added and the carbinol extracted with ether. It was then distilled. B.p., 127° at 1 mm.; yield, 75 gm.; $D_4^{25} = 0.967$.

$$[\alpha]_D^{25} = \frac{+ 1.90^\circ}{1 \times 0.967} = + 1.96^\circ. \quad [M]_D^{25} = + 3.46^\circ \text{ (homogeneous)}$$

3.216 mg. substance: 9.625 mg. CO₂ and 2.880 mg. H₂O.

C₁₂H₁₈O. Calculated. C 80.83, H 10.18

Found. " 81.60, " 10.02

Dextro-2-Phenyl-1-Bromohexane—70 gm. of 2-phenylhexanol (6), $[\alpha]_D^{25} = +1.97^\circ$, $[M]_D^{25} = +3.51^\circ$, were cooled in ice and 100 gm. of phosphorus tribromide were added. The product was heated 15 minutes on a steam bath, poured on ice, and the halide extracted with ether. It was then distilled. B.p., 133° at 1 mm.; yield, 74 gm.; $D_4^{25} = 1.201$.

$$[\alpha]_D^{25} = \frac{+2.44^\circ}{1 \times 1.201} = +2.03^\circ. \quad [M]_D^{25} = +4.90^\circ \text{ (homogeneous)}$$

5.066 mg. substance: 11.095 mg. CO_2 and 3.360 mg. H_2O .

$\text{C}_{12}\text{H}_{17}\text{Br}$. Calculated. C 59.73, H 7.11

Found. " 59.72, " 7.42

Dextro-2-Phenylhexane—A Grignard reagent was prepared from 40 gm. of 2-phenyl-1-bromohexane, $[\alpha]_D^{25} = +2.03^\circ$, $[M]_D^{25} = +4.89^\circ$, and 4 gm. of magnesium in 100 cc. of dry ether. The Grignard solution was poured on ice and hydrochloric acid. The hydrocarbon was extracted with ether, dried, and distilled from sodium. B.p., 100° at 22 mm.; yield, 21 gm.; $D_4^{25} = 0.855$.

$$[\alpha]_D^{25} = \frac{+1.68^\circ}{1 \times 0.855} = +1.96^\circ. \quad [M]_D^{25} = +3.18^\circ \text{ (homogeneous)}$$

2.597 mg. substance: 8.430 mg. CO_2 and 2.655 mg. H_2O .

$\text{C}_{12}\text{H}_{18}$. Calculated. C 88.81, H 11.19

Found. " 88.52, " 11.44

Levo-1-Amino-2-Phenylpentane—25 gm. of *n*-propylphenylpropionic acid, $[\alpha]_D^{25} = -2.32^\circ$ (homogeneous), were heated $\frac{1}{2}$ hour on a steam bath with 100 gm. of thionyl chloride. The excess thionyl chloride was distilled off under reduced pressure. The residue in the distilling flask was converted into the amide by slowly dropping into 100 cc. of cold aqueous ammonium hydroxide solution with stirring. The amide was filtered and recrystallized from 50 per cent alcohol. It was dried *in vacuo*. To the dry amide was added 1 mol of bromine. The bromo amide was poured into 200 cc. of 10 per cent potassium hydroxide and heated on a steam bath for 6 hours. It was extracted with ether, and the ether shaken with dilute hydrochloric acid. The acid solution was extracted several times with ether, then made alkaline with potassium hy-

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dioxide. The amine was extracted with ether, dried, and distilled. B.p., 90° at 3 mm.; yield, 3 gm.

$$\alpha = \frac{-0.65^\circ}{2} = -0.33^\circ \text{ (homogeneous)}$$

$$[\alpha]_D^{25} = \frac{-0.10^\circ \times 100}{1 \times 20 \times 0.895} = -0.5^\circ \text{ (in 75 per cent alcohol)}$$

$$[\alpha]_D^{25} = \frac{-0.20^\circ \times 100}{2 \times 20 \times 1.23} = -0.4^\circ \text{ (hydrochloride in 75 per cent alcohol)}$$

3.413 mg. substance: 10.010 mg. CO₂ and 2.965 mg. H₂O.

C₁₁H₁₇N. Calculated. C 80.9, H 10.5

Found. " 80.0, " 9.8

HEAT OF COMBUSTION OF ACTIVATED ERGOSTEROL*

BY CHARLES E. BILLS, FRANCIS G. McDONALD, LAMAR N. BEMILLER, GODFREY E. STEEL, AND MILDRED NUSSMEIER

(From the Research Laboratory, Mead Johnson and Company, Evansville, Indiana)

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In a previous investigation (1) we found that the heats of combustion of ergosterol and isoergosterol were essentially identical. That is to say, when ergosterol is isomerized by acids, the resulting mixture of isomers (2) is not altered as to its energy content, although it loses the property of becoming antiricketic upon irradiation. We also gave evidence, (3, 4), admittedly inconclusive, that vitamin D is related to the isoergosterols, being perhaps an intermediate form.

The determination of the heat of combustion of activated ergosterol is of importance, not only in connection with the foregoing considerations, but in view of the calculations by other workers of the energy required for activation. The early work of Pohl (5) suggested that activation may involve the addition of energy to the sterol molecule through an electron displacement. This view received support in the observation of Bills and Brickwedde (6) that activation takes place at the temperature of liquid air, at which ordinary chemical reactions fail. Measurement of the energy relations was attempted by Kon, Daniels, and Steenbock (7), who computed that 700 ergs are required for the formation of 6×10^{-8} gm. of vitamin D. Expressed as heat, this amounts to 280 calories per gm. Marshall and Knudson (8), in a similar study, concluded that decomposition makes it impossible to accumulate more than 35 per cent of vitamin D directly by irradiation.

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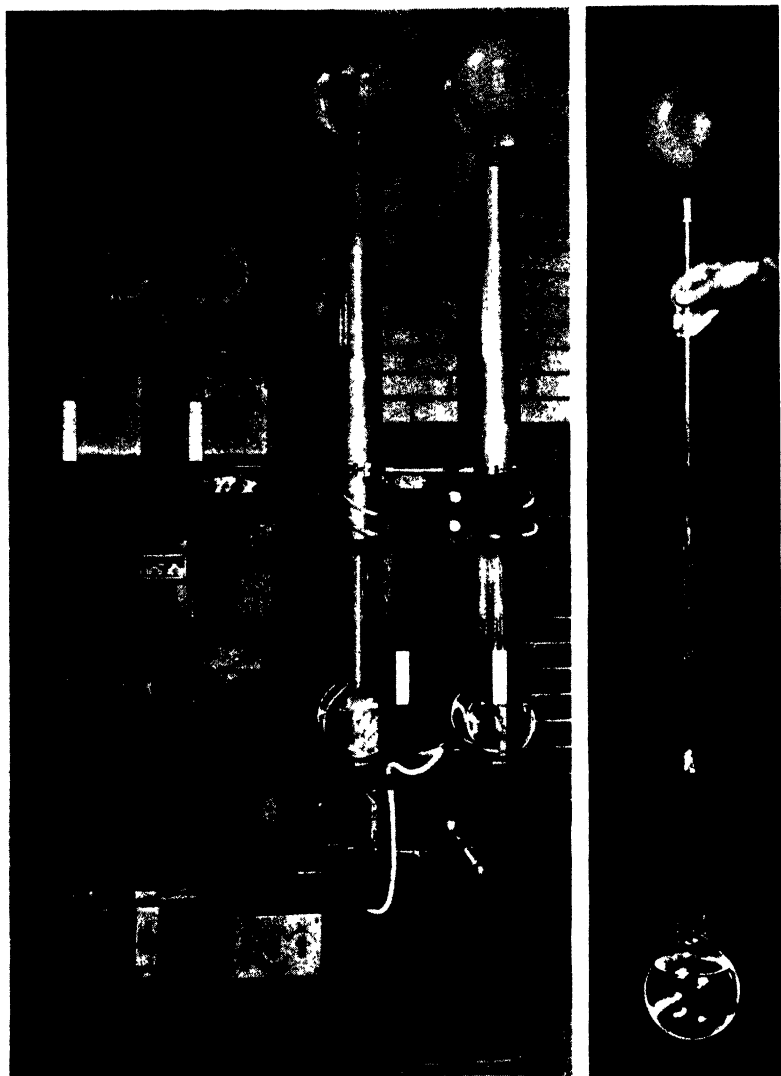


FIG. 1 Apparatus designed by one of us (C. E. B.) for the activation of ergosterol. The light source is an alternating current carbon arc operating at 40 volts, 40 amperes across the terminals. By the use of a constant current (floating secondary) transformer and a system of relays sensitive to voltage changes, the automatic feeding of the carbons is very closely regulated. The resulting constancy in electrical characteristics and hence in

Marshall and Knudson maintained that since the vitamin decomposed after prolonged irradiation with the same monochromatic wave-lengths by which it was produced, it must possess absorption bands in the same region as ergosterol itself. We once held (3) this view, but have abandoned it since we found that the entire course of formation and destruction is profoundly altered by merely changing the *solvent* (9). The whole question of the spectrographic properties of vitamin D is in the greatest confusion (4), certain preparations apparently having little, if any, absorption.

The ergosterol used in the present study was purified by repeated crystallization from alcohol-benzene 2:1. A saturated solution in boiling ether was exposed to a magnesia-cored carbon arc, until 40 per cent of the sterol was altered. Details of the irradiation technique are given with Fig. 1. The ether was evaporated, and the residue separated from unchanged ergosterol by treatment with methyl alcohol at 0°. The final product was an almost colorless resin, very sticky when warm, somewhat brittle at 0°, and without any tendency to crystallize. Tests on a preliminary batch showed it to be fairly stable in an inert atmosphere, but to change when exposed to air. In consideration of these properties we divided the examination of the product between several workers, arranging a schedule of operations so as to minimize loss of time. Parallel calorimetric, polarimetric, spectrographic, and biologic measurements were made on the fresh resin, on the resin after standing at 24° under carbon dioxide for 24 hours, after standing 3 days exposed to air, and after 45 days exposed to air.

The ergosterol, the fresh resin, and the air-exposed resins were

ultra-violet output is shown by the recording meters. In technical experience the duplicability of potency is perfect, within the limits of assay error. Equidistant from the arc four fused quartz flasks are located, each of 1 liter bulb capacity. The neck of each is greatly extended, so as to form the inner tube of a reflux condenser. Refrigerated water is circulated through the insulated jacket. 40 gm. of ergosterol and 3600 cc. of purified ether constitute a charge. CO₂ is passed through the solution to expel air, and immediately the open end of the condenser is capped with a toy balloon. Thus in the "breathing" incident to irregular boiling, the system is compelled to breathe its own breath, and ingress of air is prevented.

dehydrated for 15 minutes at 80° under high vacuum before samples were taken for examination. The heats of combustion were determined with a Parr adiabatic oxygen bomb calorimeter. The specific rotations were carried out in chloroform. The spectrographic studies were made with a Hilger quartz spectrograph, hydrogen discharge tube, and Moll recording microphotometer. The bioassays were performed with forty rats each, according to the precision technique described in our critique of the line test (10).

TABLE I
Changes Accompanying the Oxidation of Irradiated Ergosterol

Preparation	Solubility in maize oil	$[\alpha]_{D_{441}}^{20}$ in CHCl_3 degrees	λ $m\mu$	Calo- ries ₁₈ per gm.	Cod liver oil coeffi- cient
Ergosterol.	Slowly soluble	-168	260, 270, 282, 293.5	9950	0
Fresh resin.	Soluble	+ 12	270	9931	247,000
Resin under CO_2 24 hrs..	"	+ 14	272	9945	222,000
" " air 3 days.	Slowly soluble	+ 20	261	9564	253,000
" " " 45 "	Difficultly solu- ble	+ 18	253	8709	238,000
" " " 45 " then heated.	Difficultly solu- ble	+ 20	Nil	8581	126,000

The experimental findings are summarized in Table I and Fig. 2. The ergosterol showed $[\alpha]_{D_{441}}^{20} = -168^\circ$, which indicates that it was of considerably greater purity than the specimen employed in our previous determination of the heat of combustion. In keeping with this difference, the heat of combustion of the present sample was lower, being 9950 calories per gm. The photometer tracings exhibited the characteristic four absorption maxima, λ 260, 270, 282, and 293.5 $m\mu$ (11).

For the fresh resin $[\alpha]_{D_{441}}^{20} = +12^\circ$, a value typical of highly activated ergosterol preparations. The photometer tracings showed that in place of the ergosterol bands there was a single broad band of about the same intensity, λ 270 $m\mu$. This is also typical. Very careful scrutiny of the tracings by oblique viewing

revealed faint evidence of the ergosterol bands, and a digitonide determination showed 1.6 per cent of ergosterol to be present. The heat of combustion was 9931 calories per gm., a value essen-

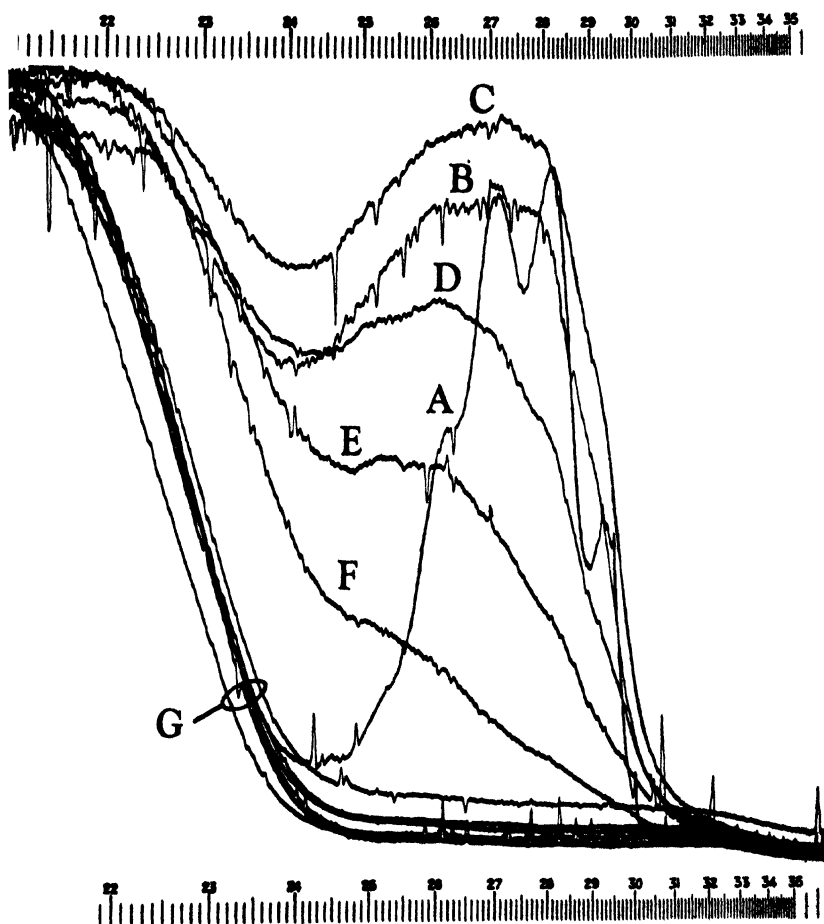


FIG. 2. Recording microphotometer tracings of the ultra-violet absorption of (A) ergosterol, (B) the fresh irradiation resin, (C) the resin after 24 hours under CO_2 , (D) the resin after 3 days in air, (E) the resin after 45 days in air, (F) the resin after 45 days in air, followed by heating, (G) the base-lines (photographic emulsion only). All concentrations 2 mg. per cc.; cell thickness 2 cm.

tially identical with that of the untreated ergosterol. The vitamin D potency, expressed as the cod liver oil coefficient, was $247,000 \pm 6$ per cent probable error.

After remaining under CO_2 for 24 hours the resin changed in certain respects, but not in others. $[\alpha]_{\text{min}}^{\text{D}}$ increased to $+14^\circ$, and λ shifted to $272 \text{ m}\mu$. Most noticeable was the *increased* intensity of absorption, which now exceeded that of ergosterol itself. Changes similar to these have been described by Windaus and Auhagen (12) for oxygen-free preparations of the resin in inert solvents. Neither the heat of combustion nor the cod liver oil coefficient was measurably altered, the former being 9945 calories per gm., and the latter, $222,000 \pm 6$ per cent probable error. Hastily, one might regard the bioassay as indicating diminished potency; actually one calculates that the values 222,000 and 247,000 differ by less than the sum of their probable errors. That no measurable diminution took place is further indicated by the assay for the following air-exposed resin, wherein the error was of opposite sign.

At this point the remaining resin was removed from CO_2 , broken up into small particles, and exposed to air for 3 days. By this treatment it gained weight, became yellow, and showed a marked decrease in its solubility in maize oil. Its specific rotation increased to $+20^\circ$. The absorption maximum shifted to $261 \text{ m}\mu$, and the absorption intensity dropped well below that of ergosterol and of the fresh or gassed resin. The heat of combustion fell to 9564 calories per gm. But in spite of these marked changes in physical properties, the vitamin D potency did not change perceptibly, for the cod liver oil coefficient was $253,000 \pm 6$ per cent probable error.

The remaining resin was exposed to air for 42 days more, making a total of 45 days. The yellow color deepened, and the substance became brittle even at room temperature. It could be dissolved in maize oil only with great difficulty. Half of the resin was taken for examination without being dehydrated, and half was dehydrated as before for 15 minutes at 80° under high vacuum. This was a wise precaution, for, as we expected, the extensively oxidized resin suffered much decomposition during the brief heating.

The undehydrated portion showed $[\alpha]_{\text{min}}^{\text{D}} = +18^\circ$. The

broad absorption band had become much less intense, giving way to general absorption, yet a maximum was still discernible, at about 253 $m\mu$. The heat of combustion showed a great decline, being 8709 calories per gm., or one-eighth less than that of the original ergosterol or fresh resin. It is the more remarkable, therefore, that the cod liver oil coefficient remained unchanged, being $238,000 \pm 8$ per cent probable error.

The dehydrated portion showed $[\alpha]_{589}^x = +20^\circ$. The absorption band almost vanished, only a suggestion of it remaining in the short ultra-violet region. Even the general absorption decreased considerably. The heat of combustion showed a small further decline, being 8581 calories per gm. The cod liver oil coefficient declined almost exactly one-half, to $126,000 \pm 8$ per cent probable error.

These findings indicate that the fresh resin was not pure vitamin D. Kon, Daniels, and Steenbock based their calculations on the assumption that the product which was formed when 6×10^{-8} gm. of ergosterol absorbed 700 ergs was the pure vitamin. Translating the potency which they reported into terms of our 5 day assay technique, one finds that their daily dosage requirement was 0.04γ .¹ The daily dosage requirement of our 250,000 X resin was 0.07γ . Our product would therefore be 57 per cent "pure" vitamin. However, this conclusion is invalid. The preparation of a resin having a cod liver oil coefficient of 425,000 (dosage requirement, 0.04γ) is an every-day commercial accomplishment, and we have obtained under certain conditions (9) a potency of 710,000 X.

¹ The translation is made as follows:

$$\frac{6\gamma}{100,000} \times \frac{10}{7} \times \frac{1}{10} \times 4.5 = 0.039 \gamma, \text{ daily for 5 days}$$

(A) (B) (C) (D)

where A is the reported total dosage administered with 50 gm. of food (a 7 day supply); B is the factor to express A on a 10 day total basis (since the technique used (Fosbinder, R. J., Daniels, F., and Steenbock, H., *J. Am. Chem. Soc.*, 50, 923 (1928)) involved keeping the rats for 10 days, feeding them a basal ration after the modified ration was consumed. Inasmuch as the normal lag is at least 3 days, the technique was essentially a 10 day procedure); C is the factor to express B on a daily basis; and D is the factor from Fig. 6 of the "Critique" (10) to translate a 10 day test into a 5 day test.

Windaus and Linsert (13) found that ergosterol irradiation products had the same molecular weight and the same unsaturation as ergosterol itself. It is reasonable to assume, therefore, that our resin was not a mixture of molecular fragments, but that it consisted of material suitable for calorimetric comparison with ergosterol. Now, if it is true that the energy required for activation remains resident in the activated material, then our fresh resin upon combustion should have yielded $0.57 \times 280 = 160$ calories per gm. more than ergosterol itself. Actually its heat of combustion remained unchanged. In other words, the energy content of the fresh resin was the same as that of the ergosterol from which it was prepared—a fact in keeping with the view of activation being a simple isomerization involving no appreciable change in energy content.

There is the possibility, of course, that vitamin D has a higher heat of combustion than ergosterol, but was present in such a minute amount in the resin that it could not be detected calorimetrically. There is also the possibility that a higher energy content of the vitamin was exactly counterbalanced by a lower energy content of the non-vitamin portion of the resin. Each of these explanations seems far fetched, even though there is good evidence that vitamin D was only a minor constituent of the resin.

Upon exposure to air for 45 days at 24° the resin showed a loss of 1241 calories per gm., or one-eighth of the value of the ergosterol. This does not mean that only one-eighth of the resin was oxidized. Since the oxidation probably affected only a small area of a large molecule, it follows that a large percentage of the resin was oxidized. This is substantiated by the marked decrease in its solubility in oil. Theoretical comparisons are not always justified, but it may be noted that a difference of one-eighth in the calories per gm. is roughly the difference observed between organic compounds derived from each other by two or three stages of oxidation. Since it is unlikely that oxidized vitamin D would be antiricketic, one is led to the opinion that the vitamin comprised but a small fraction of the total resin.

The polariscopic findings were not especially significant. It is well known that as ergosterol undergoes irradiation, its levorotation decreases and may give way to a slight dextrorotation. It is evident, however, that the dextrorotation was not due, at least

in any large measure, to vitamin D, because oxidation of the resin increased it, without increasing antiricketic potency. All that can be said is that the mean $[\alpha]$ of the components of the resin was positive in sign, but from this nothing can be inferred as to the specific rotation of the vitamin itself.

It would appear that nearly every investigator of activated ergosterol has attributed to vitamin D the absorption maxima of the preparation he investigated. In our preparations the maximum moved about, without any change in vitamin potency. In the fresh resin it was at 270 $m\mu$, in the resin preserved under CO_2 it was at 272, and in the air-exposed resins it was at 261 and 253 $m\mu$. Obviously, none of these maxima was due to the vitamin. The resin which was heated after prolonged exposure to air retained half of its potency while losing most of its absorption. All this is additional evidence that the vitamin does not absorb. To explain its decomposition after prolonged irradiation, we venture the suggestion that the strongly absorbing, non-vitamin constituents of the resin become converted by the absorbed radiations into substances which are chemically destructive of the vitamin. In a later report we shall present evidence which indicates that the absorbing substances discussed here are formed from ergosterol simultaneously with the vitamin.

At the completion of this work we learned of the preparation of a crystalline antiricketic substance, calciferol, by Angus, Askew, Bourdillon, Bruce, Callow, Fischmann, Philpot, and Webster (14, 15). Windaus (15), by the use of maleic anhydride, has obtained a preparation apparently similar to that which the English workers got by fractional condensation of the distilled resin.

It is most interesting to compare the properties of calciferol with those of the resin. By use of the data given previously (10), it is possible to translate antiricketic values obtained by different methods of assay, and in so doing we observe that the potency of calciferol is slightly less than that of our resin. The English authors found it to be not more potent than the resin from which it was prepared, and they concluded that there are probably at least two forms of vitamin D, it being uncertain whether calciferol occurs in the resin or is produced by the subsequent action of heat.

Assuming for the moment that calciferol does not owe its potency to a superpotent contaminant, one may regard our work as evidence for the existence of more than one form of vitamin D. While the potency of the resin which we obtained with magnesia carbons was not greatly different from that of calciferol or its parent resin, the fact that in other experiments we have prepared resins of much greater potency proves that in them the antiricketic activity was not due entirely to calciferol. The substitution of iron for magnesium in the carbons raises the potency to 425,000 X, the difference being due presumably to the preponderance of the more effective short waves in the iron spectrum.

Other evidence of the multiple nature of vitamin D was seen in the fact that chickens required many times as much irradiated ergosterol as cod liver oil, in the form of solutions equipotent for rats (16). This can be demonstrated even more strongly with the vitamin prepared from ergosteryl acetate (unpublished observations). The chicken and rat experiments will be continued, with a new preparation of vitamin D prepared chemically, without the agency of ultra-violet rays.

There is no reason to believe that the resins used by the English and German workers differed fundamentally from ours. Those resins contained, or rather yielded, nearly 50 per cent of calciferol. This fact, considered with the evidence we presented as to the minuteness of the potent fraction, gives rise to troublesome thoughts regarding the purity of the crystalline preparations. Although the English workers, in carefully planned experiments, were unable to attribute the potency of calciferol to contamination, additional evidence to the contrary is still to be desired.

SUMMARY

Parallel calorimetric, polarimetric, spectrographic, and biologic measurements were made on ergosterol, and on its irradiation product after progressive oxidation. While critical interpretation of the findings must be tentative in some instances, the following conclusions are offered:

1. The heat of combustion of ergosterol is lower than previously reported, being 9950 calories₁₅ per gm.
2. The heat of combustion of fresh irradiation resin is essentially identical with that of ergosterol, there being no evidence of a

higher energy level in the vitamin. This fits with the hypothesis that vitamin D is a simple isomer of ergosterol.

3. In oxidized resin, potency remains, while heat of combustion falls markedly, this indicating that vitamin D does not constitute the major part of the resin.

4. Past attempts to determine the energy requirements of activation appear to have little value, in view of the evidence that the vitamin is but a minor constituent of the resin.

5. There is evidence that vitamin D may exist in more than one form.

6. In the resin, vitamin D either has no absorption, or is present in an amount too minute for its absorption to be detected.

7. The question as to the purity of the recently described crystalline antiricketic preparations is involved in the estimation of the quantity of vitamin D present in active resins.

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THE MICRO DETERMINATION OF FIXED BASES, CALCIUM, AND SULFATES IN URINE

By WILLIAM S. HOFFMAN*

(From the Department of Medicine, Rush Medical College, the University of Chicago, Chicago)

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In metabolic studies requiring the examination of a large number of specimens of urine, the determination of the inorganic constituents is a difficult laboratory problem. The accepted methods of determination, even when accurate, either require such large quantities of urine or are so time-consuming that their utilization in the study of frequent, say 2 hour, specimens is impossible. Besides, each method usually involves an independent preliminary preparation of the urine for the individual determination, which not only means an expenditure of time but usually a loss of a quantity of urine.

In particular, the available methods for the determination of the total fixed bases and of the calcium in urine present such difficulties. Fiske's (1) method for fixed base, though sound in principle and capable of giving accurate results, is a protracted, bothersome procedure. Again, those methods for the determination of calcium directly in urine, like that of McCrudden (2) or its modifications, in their avoidance of the hazardous ashing of the urine, seek a dubious accuracy by using 100 to 200 cc. of urine and allowing 24 hours for the precipitation of the calcium oxalate.

It is the purpose of this paper to present a new scheme for the determination of fixed bases, calcium, and sulphates in the urine, which has the proposed advantage of simplicity as well as of accuracy, rapidity, and requiring only a small quantity of urine.

The principle underlying the scheme is the preparation of a protein-free, phosphate-free filtrate, which contains all the inor-

* National Research Council Fellow in Medicine; under the supervision of Dr. Wilber E. Post.

ganic constituents in their original proportions, and which can be used directly for the determination of the various constituents. Though this paper concerns itself with the determination of fixed bases, calcium, and sulfate, the filtrate can be utilized also for the micro estimation of potassium, sodium, and magnesium.

Procedure

15 cc. of urine are measured into a large Pyrex test-tube of 60 cc. capacity accurately marked for 25 cc. The urine is made faintly acid to methyl red by the addition of either 10 per cent acetic acid or 10 per cent ammonium hydroxide. 2 cc. of a 10 per cent solution of ferric chloride in 0.2 N HCl are slowly added and followed by 4 cc. of 5 per cent ammonium acetate. The solution is made up to 25 cc. and carefully heated to boiling in a free flame, with constant agitation. At the boiling point, a voluminous precipitate is present which contains all the phosphates as ferric phosphate (and organic phosphates), all the excess iron as ferric basic acetate, and any protein, fat, or debris that may have been present in the urine, along with a good proportion of the urinary pigment. This is filtered immediately through a small ash-free filter paper. About 22 cc. of filtrate are obtained. The precipitate should have the brick-red color of the basic ferric acetate. If it is brownish white, the quantity of ferric chloride was insufficient to precipitate all the phosphate. This circumstance occurs only when extremely concentrated specimens of urine are being examined. In such cases, it is better to start with 10 cc. of urine, the remaining procedure being the same.

Determination of Fixed Bases—2 cc. of the filtrate, equivalent to 1.2 cc. of urine, are transferred to a 25 cc. platinum crucible and treated with 1 cc. of 4 N H_2SO_4 . This solution is evaporated on a steam bath until charring occurs, which is accomplished in about 30 minutes. The crucible is now transferred to a Rogers ring burner equipped with a platinum wire sling for the crucible, where the ashing is completed. The flame is first played above the crucible and then over the rim. As the charred mixture reaches the boiling point of the sulfuric acid, it rises in the bottom of the crucible to form a thick black foam, from the surface of which the sulfuric acid evaporates without spattering. With constant watching and with courageous, but at the same time discreet,

raising of the crucible, the ashing can be completed in 8 to 10 minutes. When all the organic matter has been destroyed, the crucible is kept at red heat throughout for 1 minute, by turning the flame of the Rogers burner on to full height. Every trace of free H_2SO_4 is thus removed. The ash always contains a small amount of iron oxide, which, however, does not interfere with the determination.

After the crucible has cooled, the sulfates are determined by the indirect titration method of Stadie and Ross (3). Exactly 15 cc. of water are measured into the crucible, followed by 2 cc. of a standardized benzidine solution in HCl .¹ The mixture is thoroughly stirred with a rubber-tipped glass stirring rod until it is certain that all the inorganic sulfate has been dissolved and reprecipitated as benzidine sulfate. The latter is filtered off on an ashless filter paper. 15 cc. of the filtrate are titrated to neutrality to phenol red with 0.02 N NaOH . This titration value, when corrected for the volume used and subtracted from the equivalent of 2 cc. of benzidine solution, gives the amount of acid in the precipitated benzidine sulfate. This is equivalent to the amount of base originally present. The calculation of the total base is illustrated in the following example.

An aliquot equal to 1.2 cc. of urine was used for the ashing. 2 cc. of benzidine solution required 21.00 cc. of 0.02 N NaOH . 15 cc. of the final filtrate from benzidine sulfate (the total being 17 cc.) required 7.10 cc. of 0.02 N NaOH .

$$[21.00 - (7.1 \times \frac{1}{15})] \times \frac{1}{2} \times \frac{1}{10} = 2.16 \text{ cc. } 0.1 \text{ N fixed base per cc. urine}$$

Determination of Calcium.—The original filtrate from the ferric phosphate and basic acetate is used for the direct precipitation of calcium oxalate. 10 cc. of this filtrate (representing 6 cc. of urine) are transferred to a 15 cc. graduated centrifuge tube. Exactly 2 cc. of 4 per cent ammonium oxalate are added. The resulting mixture will be just about neutral or faintly alkaline to methyl

¹ In a 250 cc. volumetric flask, 4 gm. of pure benzidine are added to 45 cc. of N HCl diluted with water to 150 cc., and agitated until dissolved. The solution is made up to mark and filtered. 2 cc. portions are standardized by titration with 0.02 N NaOH with phenol red as an indicator. About 20 cc. of 0.02 N NaOH are usually required. The benzidine solution should be refiltered every few days and restandardized.

red, the ideal pH for the precipitation of calcium oxalate. At the end of an hour, the mixture is centrifuged for 10 minutes and the supernatant fluid carefully poured off by decantation. (This fluid can be used for the estimation of magnesium by the method of Denis (4). 10 cc. of it represents 5 cc. of urine.) The precipitated calcium oxalate is washed with 4 cc. of a saturated solution of calcium oxalate in 0.5 per cent NH_4OH (5), and the supernatant fluid removed by the centrifugation and decantation. The washing is repeated once. The oxalate is dissolved in 4 cc. of 5 per cent H_2SO_4 , heated to 70° in a water bath, and titrated with 0.02 N KMnO_4 from a micro burette. The amount of calcium is easily calculated, since 2.5 cc. of 0.02 N KMnO_4 are equivalent to 1 mg. of calcium.

Determination of Sulfate.—The original filtrate, since it is free of phosphate, can be used directly for the determination of sulfate by the method of Fiske (6) which has here been modified in the direction of greater simplicity. 5 cc. of the filtrate (representing 3 cc. of urine) are transferred to a 50 cc. beaker, diluted with 10 cc. of water, and treated with 2 cc. of benzidine chloride solution. The standardized benzidine solution mentioned in the determination of fixed bases may be used. The precipitate is filtered off on a 7 cm. ashless filter paper, the filtrate being used for the quantitative transferal of the precipitate to the filter. If the filter is first made wet with acetone, the filtration is found to proceed rapidly. The precipitate is washed four times with 2 cc. portions of pure acetone, complete draining being allowed at the end of each washing. This washing is best accomplished by dropping the acetone from a dropping bottle, so that every portion of the funnel, paper, and precipitate is thoroughly washed free of the benzidine chloride solution. The precipitate is now transferred to a small Erlenmeyer flask by puncturing a hole in the paper without removing it from the funnel, and washing the precipitate into the flask with hot water from a wash bottle. Care, of course, must be taken to see that every trace of precipitate is washed into the flask. The flask is now heated to boiling and the solution titrated with 0.02 N NaOH to neutrality to phenol red at the boiling point. Each cc. of 0.02 N NaOH is equivalent to 0.32 mg. of S as sulfate. A blank determination should be made on the reagents used.

Total sulfate can also be determined from the original filtrate by

the method of Fiske (6). 5 cc. of the filtrate are treated with 3 cc. of 3 N HCl in a 50 cc. beaker and evaporated to dryness on a water bath. About 15 minutes longer are allowed to remove all of the HCl. The residue is then dissolved in 15 cc. of water, treated with 2 cc. of benzidine solution, and the sulfates determined as described above.

Comment

It is readily seen that in the method outlined here for fixed bases, the principles of the Fiske method are retained. The chief difference lies in the omission of the preliminary Neumann combustion of the urine with sulfuric and nitric acids. The procedure is troublesome, time-consuming, and unnecessary, since even in the Fiske method ashing is finally done in a platinum crucible. Phosphates can be removed by the iron method just as readily from the original urine as from the carbon-free solution by the Neumann combustion. That no bases other than iron are precipitated is demonstrated by the classic use of the iron method for the removal of phosphate before the metals of group III are precipitated in the ordinary scheme of qualitative analysis. This fact is further attested to in Tables I, II, IV, and V of this paper. With the use of the Neumann procedure, too, there is an appreciable blank because of the large quantities of H_2SO_4 and HNO_3 used.

In the Fiske method, the solution containing the ferric phosphate is heated to boiling, then made up to mark, filtered, and an aliquot of the cooled filtrate used. Fiske allows empirically 1 per cent for the error involved in the contraction of the solution on cooling. It is not clear why Fiske utilizes this technique, when it is obviously more correct to make the solution up to the mark in the cold, heat to boiling, filter, and take an aliquot from the cooled solution. Experiments on pure solutions of inorganic substances demonstrate that the error is more than 1 per cent; it is nearer 3 per cent. In Table I, therefore, when the results of the proposed method are compared with those of the Fiske method, the latter results are obtained with the modification of the method as used here, so that the results may be comparable.

The indirect titration method of Stadie and Ross has been used here instead of the direct determination of the sulfate by the method of Fiske, because it is more rapid and gives more consist-

ently accurate results, since it avoids the necessity for washing the precipitate and for titrating at the boiling point. The method is admirably suited here, for there are no phosphates present. Phosphates interfere not only because they are incompletely precipitated as benzidine phosphate, but also because their buffer action prevents the complete precipitation of the sulfate and prevents accurate titration of the filtrate. These facts were recognized in unsuccessful attempts on the part of the author to apply the method of Stadie and Ross to the determination of sulfates in urine.

TABLE I

Comparison of Fixed Base Determinations by Author's Method with Those by Fiske's Method

Determination No.	Fixed base per 100 cc. urine	
	Author's method	Fiske method
	cc. 0.1 N	cc. 0.1 N
1	275	278
2	202	205
3	181	186
4	221	226
5	141	143
6	216	215
7	126	128
8	144	150

Of course, the greatest advantage of the proposed method for fixed bases lies in the fact that the phosphate-free, protein-free filtrate can be used directly for the micro determination of calcium and sulfate. Also, since the filtrate from the calcium oxalate precipitate can be used for the estimation of magnesium, and since potassium can be determined from an aliquot of the dissolved ashing, and since sodium is probably just as well determined by difference as by direct estimation, it will be recognized that with 15 cc. of urine, it is possible to determine all the basic constituents as well as the sulfates.

The accurate determination of calcium in an aliquot representing only 6 cc. of urine is possible because there is nothing present in the solution to interfere with the complete precipitation of the calcium oxalate. The precipitate settles out within 15 minutes

as a clean crystalline mass. 1 hour is more than ample time to make sure that there is no further precipitation. The results obtained agree well with those from ashed samples, and are higher than those by the method of McCrudden or of Shohl and Pedley

TABLE II

Comparison of Calcium Determinations by Author's Micro Method with Those by Macro Methods

Determination No.	Ca per 100 cc. urine			
	Author's method	Shohl and Pedley method	McCrudden method	Ashing method
	mg.	mg.	mg.	mg.
1	13.0			12.5
2	12.3		12.1	
3	10.4	9.5		10.0
4	5.2		4.7	5.4
5	12.8	12.2		12.5
6	13.3	12.6	12.2	13.1

TABLE III

Comparison of Sulfate Determinations by Author's Method with Those by Fiske's and Gravimetric Method of Folin

The values are expressed as mg. of S per 100 cc. of urine.

	Determination No.	Author's method	Fiske method	Gravimetric method
Inorganic sulfate	1	42.2	43.2	42.9
	2	20.8	20.3	20.2
	3	85.3	85.8	85.4
	4	21.4	22.4	
	5	50.0	50.1	
	6	73.1	72.5	
Total sulfate	1	27.7	28.3	
	2	58.2	59.5	
	3	64.0	66.1	

(7), even though 18 to 24 hours are allowed for the precipitation in these methods (see Table II).

The determination of sulfates as benzidine sulfate, though theoretically simple, is attended with a number of difficulties, some of which are not easily explicable. The inaccuracy of the original Rosenheim and Drummond (8) procedure was demon-

TABLE IV

Recovery of K_2SO_4 Added to Urine

In Experiment A increments of K_2SO_4 were added to measured portions of the same urine, so that, calculated on the basis of 100 cc., the portions contained 8, 16, 24, and 32 mg. of K, respectively, more than the original urine. All these portions, along with the original urine, were analyzed by the author's method for fixed base, sulfate, and potassium. The potassium was determined from an aliquot of the ashing, by the method of Jacobs and Hoffman (9). Experiment B is a similar one on another urine sample in which the increments were 24, 48, 72, and 96 mg. of K per 100 cc. of urine respectively.

Determination No	Experiment A				Experiment B			
	Fixed base	Added base	Total base		Fixed base	Added base	Total base	
			Calculated	Found			Calculated	Found
	cc. 0.1 N	cc. 0.1 N	cc 0.1 N	cc 0.1 N	cc. 0.1 N	cc 0.1 N	cc 0.1 N	cc 0.1 N
0	101.7				134.8			
1	101.7	2.05	103.75	104.0	134.8	6.1	140.9	140.5
2	101.7	4.10	105.8	106.5	134.8	12.2	147.0	146.2
3	101.7	6.15	107.85	108.7	134.8	18.3	153.1	153.8
4	101.7	8.20	109.9	109.7	134.8	24.4	159.2	160.3
Determination No.	Inorganic S	Added S	Total inorganic S		Inorganic S	Added S	Total inorganic S	
			Calculated	Found			Calculated	Found
	mg	mg	mg	mg	mg.	mg.	mg	mg
0	31.79				32.32			
1	31.79	3.28	35.07	34.34	32.32	9.84	42.16	43.20
2	31.79	6.56	38.35	37.23	32.32	19.68	52.00	52.59
3	31.79	9.84	41.63	40.53	32.32	29.52	61.84	62.93
4	31.79	13.12	44.91	42.88	32.32	39.36	71.68	71.47
Determination No.	K	Added K	Total K		K	Added K	Total K	
			Calculated	Found			Calculated	Found
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
0	109.4				178.0			
1	109.4	8	117.4	117.9	178.0	24	202.0	204.1
2	109.4	16	125.4	124.9	178.0	48	226.0	226.2
3	109.4	24	133.4	135.3	178.0	72	250.0	257.6
4	109.4	32	141.4	140.7	178.0	96	274.0	277.5

strated by Fiske, who proposed a method in which the sulfates are determined in a solution from which the phosphate has been removed as magnesium ammonium phosphate. Even here, accurate results are obtained only if the solution is made neutral to brom-phenol blue (pH about 4). In the method described here, high results are obtained if the benzidine solution is added to the undiluted filtrate. However, if the solution is diluted with twice its quantity of water, results are obtained which agree well with those by the Fiske method and with those by the gravimetric barium sulfate method (see Table III). It was found unnecessary to adjust the pH to neutrality to brom-phenol blue. The original filtrate has a pH about 5, and is buffered by the presence of acetate. This phosphate-free solution when diluted gives the same values as

TABLE V
Recovery of Ca Added to Urine

The results are expressed as mg. per 100 cc. of urine.

Determination No	Ca	Added Ca	Total Ca		Ca	Added Ca	Total Ca	
			Calculated	Found			Calculated	Found
0	25.13				10.53			
1	25.13	2.5	27.63	27.66	10.53	4	14.53	14.84
2	25.13	5.0	30.13	30.36	10.53	8	18.53	18.16
3	25.13	7.5	32.63	32.89	10.53	12	22.53	22.48
4	25.13	10.0	35.13	35.93	10.53	16	26.53	26.26

those obtained from the phosphate-free filtrate of Fiske adjusted to pH 4.

Tables IV and V present data showing how accurately added bases may be recovered from urine by the author's methods. In the experiments presented in Table IV, K_2SO_4 was added to specimens of urine and was recovered as fixed base, as sulfate, and as potassium. In a similar way, Table V describes experiments showing the accurate recovery of known amounts of calcium added to specimens of urine.

SUMMARY

A new system for the determination of the individual and total fixed bases as well as sulfates in urine is presented, in which all the

analyses are made from a single protein-free, phosphate-free filtrate prepared from the urine.

The methods for total fixed base, calcium, and sulfates have been modified so that they have the advantage of simplicity, accuracy, rapidity, and of requiring only a small quantity of urine.

Data are presented confirming the accuracy of the proposed methods.

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LACTATE CONCENTRATION IN THE BLOOD OF THE RABBIT AFTER INJECTION OF SODIUM LACTATE

BY I. A. PARFENTJEV, V. D. SUNTZEFF, AND B. F. SOKOLOFF

(From the Department of Pathology, Washington University School of Medicine, St. Louis)

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In our present work we have studied the level of lactic acid in the blood after injection of sodium lactate. We considered the determination of the highest possible concentration of lactic acid in blood especially important. This high concentration could be reached by treating animals with sodium lactate. We also wished to estimate the length of time during which the increased level of lactic acid in the blood could be maintained. Most of our experiments described below were performed with rabbits. We determined the lactic acid by the method of Mendel and Goldscheider as modified by Fuchs (4). Blood was taken for analysis from the hearts of the rabbits.

Table I shows the amount of lactic acid found in the blood of normal rabbits before injection of sodium lactate.

Using the above method we estimated that the normal amount of lactic acid in the blood of grown rabbits was about 0.33 mg. per 1 cc. of blood, or 0.03 per cent. The rabbits were then injected with sodium lactate prepared by neutralizing racemic lactic acid with sodium hydroxide. By adding to 100 cc. of 85 per cent lactic acid 240 cc. of 4 N sodium hydroxide we obtained a solution (pH 5) corresponding almost exactly to a pure sodium lactate solution. Another solution of pH 7 was prepared by adding to 100 cc. of 85 per cent lactic acid 280 cc. of 4 N sodium hydroxide. In this solution there was an obvious excess of sodium hydroxide. These solutions were diluted with distilled water in order to obtain different concentrations for the tests. The concentrations given below represent the amount of free lactic acid.

Sodium lactate was injected into the ear vein, subcutaneously,

and was also administered by mouth. By previous determination we had found that rabbits weighing from 2.0 to 2.5 kilos could tolerate, without danger, the injection of the following amounts of lactic acid in the form of sodium lactate at pH 7:

Intravenously.....	about 10 cc. of 10 per cent lactic acid
Subcutaneously	" 30 " " 10 " " " "
Orally.....	" 30 " " 20 " " " "

Larger amounts produced shock and often the death of the animals. Protocols of some of our experiments are given below.

TABLE I
Lactic Acid in Blood of Rabbits before Injection of Sodium Lactate

Rabbit No.	Weight	Lactic acid per cc. of blood
	gm.	mg.
3	2400	0.08
9	2840	0.12
11	2640	0.20
12	2250	0.25
8	2450	0.25
6	3180	0.25
2	2040	0.30
10	2740	0.35
7	3160	0.50
5	1980	0.50
1	2070	0.60
4	2550	0.65

Rabbit 1—Weight 2070 gm. 1 cc. of blood contains 0.60 mg. of lactic acid. 20 cc. of 10 per cent lactic acid, pH 7, were injected into the ear vein. 15 minutes after injection 1 cc. of blood contained 2.00 mg. of lactic acid. While the rabbit was being bled from the heart it died.

Rabbit 2—Weight 2040 gm. 1 cc. of blood contains 0.30 mg. of lactic acid. 10 cc. of 10 per cent lactic acid, pH 7, were injected into the ear vein. 1 cc. of blood contained 1.00 mg. of lactic acid 15 minutes after injection, 0.30 mg. 6 hours after.

Rabbit 3—Weight 2400 gm. 1 cc. of blood contains 0.08 mg. of lactic acid. 10 cc. of 10 per cent lactic acid, pH 7, were injected into the ear vein. 1 cc. of blood contained 1.00 mg. of lactic acid 5 minutes after injection, 0.08 mg. 2 hours after, and 0.08 mg. 3 hours after. After 2 days we repeated the experiment with this rabbit. 0.08 mg. of lactic acid was found in 1 cc. of blood taken for analysis. 15 cc. of 10 per cent lactic acid, pH 7, were injected.

1 cc. of blood contained 2.00 mg. of lactic acid 10 minutes and 30 minutes after injection. Soon after the last sample was taken the rabbit died.

Rabbit 4—Weight 2550 gm. 1 cc. of blood contains 0.65 mg. of lactic acid. 10 cc. of 10 per cent lactic acid, pH 7, were injected into the ear vein. 1 cc. of blood contained 1.75 mg. of lactic acid 5 minutes after injection, 1.50 mg. 30 minutes after, 0.80 mg. 1 hour after, and 0.25 mg. 2 hours after. After 4 days a new experiment was started with this rabbit. In 1 cc. of blood taken for analysis we found 0.25 mg. of lactic acid. We injected subcutaneously 50 cc. of 10 per cent lactic acid, pH 7. 1 cc. of blood contained 0.60 mg. of lactic acid 30 minutes after injection, 1.00 mg. 1 hour after, 1.60 mg. 2 hours after, and 1.60 mg. 3 hours after. Soon after taking the last sample the rabbit died.

Rabbit 5—Weight 1980 gm. 1 cc. of blood contains 0.50 mg. of lactic acid. 50 cc. of 10 per cent lactic acid, pH 7, were injected subcutaneously. 1 cc. of blood contained 0.90 mg. of lactic acid 3 hours after injection, 1.50 mg. 6 hours after, and 2.00 mg. 8 hours after. Soon after the last sample was taken the rabbit died.

Rabbit 6—Weight 3180 gm. 1 cc. of blood contains 0.25 mg. of lactic acid.

Rabbit 7—Weight 3160 gm. 1 cc. of blood contains 0.50 mg. of lactic acid. 30 cc. of 10 per cent lactic acid, pH 7, were injected subcutaneously. 1 cc. of blood contained 0.50 mg. of lactic acid 2 hours after injection, 0.75 mg. 4 hours after, and 0.60 mg. 6 hours after.

Rabbit 8—Weight 2450 gm. 1 cc. of blood contains 0.25 mg. of lactic acid. 30 cc. of 10 per cent lactic acid, pH 7, were injected. 1 cc. of blood contained 1.00 mg. of lactic acid 6 hours and 9 hours after injection.

Rabbit 9—Weight 2840 gm. 1 cc. of blood contains 0.12 mg. of lactic acid. 30 cc. of 10 per cent lactic acid, pH 7, were injected subcutaneously. 24 hours after injection 1 cc. of blood contained 0.12 mg. of lactic acid. After 3 days we repeated the experiment with this rabbit. 1 cc. of blood taken for analysis contained 0.12 mg. of lactic acid. 30 cc. of 10 per cent lactic acid, pH 7, were injected subcutaneously. 1 cc. of blood contained 0.25 mg. of lactic acid 12 hours after injection, 0.12 mg. 24 hours after.

Rabbit 10—Weight 2740 gm. 1 cc. of blood contains 0.35 mg. of lactic acid. 30 cc. of 20 per cent lactic acid, pH 7, were administered orally. 24 hours after administration 1 cc. of blood contained 0.20 mg. of lactic acid.

Rabbit 11—Weight 2640 gm. 1 cc. of blood contains 0.25 mg. of lactic acid. 30 cc. of 20 per cent lactic acid, pH 7, were administered orally. 1 cc. of blood contained 0.50 mg. of lactic acid 12 hours after administration, 0.20 mg. 24 hours after.

Rabbit 12—Weight 2250 gm. 1 cc. of blood contains 0.25 mg. of lactic acid. 30 cc. of 20 per cent lactic acid, pH 7, were administered orally. 1 cc. of blood contained 0.85 mg. of lactic acid 3 hours after administration, 1.50 mg. 6 hours after.

The experiments are summarized in Charts 1 to 3.

DISCUSSION

In the literature we find different statements concerning the amount of lactic acid in rabbit blood. Very careful determinations were made on a large number of animals by Kawamura (6). The amount of lactic acid which he found in the blood of normal rabbits is nearly that found by us. Kawamura reports an average of 0.2 mg. of lactic acid per 1 cc. of blood with a variation of



CHART 1. Level of lactic acid in blood after administration of sodium lactate by stomach. 30 cc. of 20 per cent sodium lactate at pH 7 were introduced orally. Weight of rabbits, about 2.5 kilos. The ordinates represent lactic acid measured in mg. per cc. of blood; the abscissas, time in hours after administration.

0.098 to 0.45 mg. Cori, Cori, and Buchwald (3) found in rabbit blood 0.059 to 0.10 mg. of lactic acid per 1 cc. of blood. Laufberger (7) claims that he found a slightly larger amount of lactic acid in rabbit blood, namely 0.31 to 0.96 mg. per 1 cc. of blood. Collazo and Morelli (1) report a more pronounced variation in the amount of lactic acid in rabbit blood. The average amount of lactic acid which they found in 1 cc. of blood was 0.51 mg. and the variations were from 0.13 to 1.0 mg. Considerably higher amounts of lactic acid in rabbit blood were shown by Valentin (9); namely, 0.75 to 1.28 mg. of lactic acid per 1 cc. of blood.

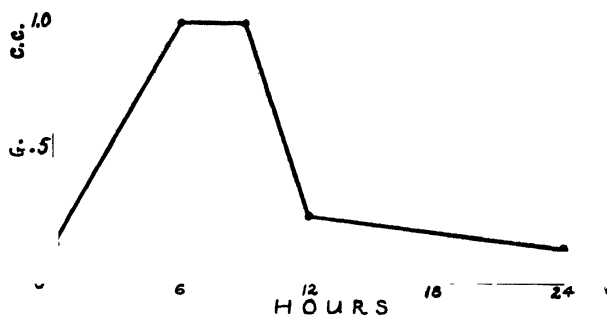


CHART 2. Level of lactic acid in blood after subcutaneous injection of sodium lactate. 30 cc. of 10 per cent sodium lactate at pH 7 were injected subcutaneously. Weight of rabbits, about 2.5 kilos. The ordinates represent lactic acid measured in mg. per cc. of blood; the abscissas, time in hours after injection.

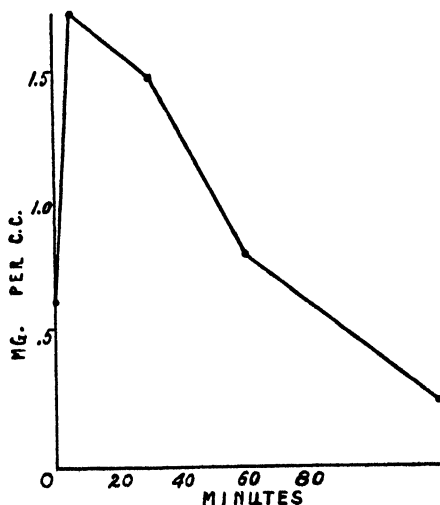


CHART 3. Level of lactic acid in blood after intravenous injection of sodium lactate. 10 cc. of 10 per cent sodium lactate at pH 7 were injected. Weight of rabbits, 2.5 kilos. The ordinates represent lactic acid measured in mg. per cc. of blood; the abscissas, time in minutes after injection.

From the above data it can be seen that the average amount of lactic acid in rabbit blood determined by several different authors was approximately the same; the noted variations are apparently

due partly to the different methods which were used for lactic acid determination and partly to the changes in the amount of lactic acid in connection with physiological conditions of animals.

Very important work on lactic acid has been done by Cori and Cori (2). Their experiments with rats showed that by oral administration or subcutaneous injections of sodium *l*-lactate 30 per cent of the amount absorbed was excreted in the urine, while no excretion occurred in the case of sodium *d*-lactate. The latter is transformed into glycogen. Fuerth and Engel (5) have reported on the assimilation of racemic lactic acid. They showed that by feeding control animals with fermented lactic acid 20 to 30 per cent of the absorbed lactic acid was excreted in the urine.

In studying the available literature on this subject, we did not find any report on the highest level of lactic acid in blood obtained by injecting lactic acid preparations, or the period of time during which the lactic acid introduced can be maintained in the organism.

SUMMARY

1. Using different methods of administering sodium lactate, we were able to increase, without danger, the level of lactic acid in the blood of rabbits up to 1.5 to 1.7 mg. per 1 cc., which corresponds to from 5 to 6 times the original level in the blood. By using larger amounts of sodium lactate we observed a level of about 2 mg. of lactic acid per 1 cc. of blood. In these cases, however, the animals died. It seems that 2 mg. of lactic acid per 1 cc. of blood are the lethal dose of this compound.

2. When intravenous injections are used lactic acid disappears very rapidly from the blood; *i.e.*, 1 to 2 hours after injection.

3. When sodium lactate was administered subcutaneously or by mouth, the highest concentration of lactic acid in blood was obtained. In both cases the increase in concentration of lactic acid in the blood was observed almost always during the first 12 hours after the treatment. The maximum increase was reached between 4 to 9 hours after each administration of sodium lactate.

4. In some experiments, after application of sodium lactate following the initial increase of lactic acid in the blood, there was noted a decrease of lactic acid which reached a level below that of normal.

5. Lactic acid in the form of sodium lactate at pH 7 could be

injected without danger to rabbits in the following amounts shown in gm. per kilo of weight of the animal: intravenously 0.5 gm., subcutaneously 3.0 gm., and orally 6.0 gm.

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THE COMPOSITION OF VIXEN MILK

By E. GORDON YOUNG AND G. A. GRANT

(From the Department of Biochemistry, Dalhousie University, Halifax, Canada)

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In certain sections of Eastern Canada, notably Prince Edward Island, fox farming is practised extensively. Here the problem of rearing a litter of fox puppies by artificial means arises occasionally. We are informed that the most successful means of nursing small foxes deprived of their mother is by introducing them into a litter of kittens, from which they are almost indistinguishable. If the mother cat accepts them the growth of the foxes is usually good. This practise is not always possible and recourse is taken to feeding artificially by cow's milk. On the latter, however, the mortality is high. The scientific explanation of these observations seemed to be most probably in the composition of the milk of the fox. A careful search of the literature, however, revealed not a single analysis when these analyses were undertaken 2 years ago.

Through the kind cooperation of Mr. Champion Holland of Albany, Prince Edward Island, and Mr. G. Ennis Smith, Superintendent of the Experimental Fox Ranch at Summerside, Prince Edward Island, several samples of fox milk, preserved with thymol, were sent to our laboratory. The composition of these samples is given below in so far as the amounts permitted of analysis. The milk can only be obtained in the spring and from an animal that is relatively tame.

The first sample was taken at a single milking on May 8, from a healthy, well furred, medium silver fox that had given birth to four puppies on April 11. In appearance it was of a slightly yellowish color, of a thick, creamy consistency, and of a faint, asphalt-like odor. It settled into three distinct layers: a heavy, yellowish, upper layer, a white, thinner, middle layer, and a watery,

wey-like, lower layer. Examination under the microscope revealed fat globules varying greatly as to size.

The second sample was a composite of three fractions obtained from three silver foxes on May 14. These animals had given birth to litters on or about April 17. The sample was thus representative of the milk after 4 weeks of lactation. Its characteristics were similar to the first sample except that it was white in color and about the consistency of cow's milk.

The third sample was obtained from a vixen on the 3rd day of lactation. It was slightly yellow in color, very viscous, and possessed the characteristic, asphalt-like odor.

For the most part the methods as given in Leach (9) were followed. Protein and lactose were determined on separate portions. The material from the estimation of the specific gravity was used for the estimation of total solids, and the residue from this extracted in a Soxhlet apparatus for the determination of fat, and the residue from this ignited for the determination of the ash. The latter was extracted with dilute acid, made up to definite volume, and aliquot portions used for the estimations of calcium and phosphorus.

The results of the analyses and the methods employed are given in Table I. We have included two analyses by Laxa (7, 8) which were discovered in the literature after the appearance of the 1930 index volume to Chemical Abstracts. There is to be noted a close agreement in the values obtained with our Samples 1 and 2 and the second sample analyzed by Laxa. The period of lactation is about the same for all three. These values have been used to express the general average for the composition of the milk of the vixen and represent the average from five animals. Our Sample 3 was a late colostrum and is distinctly different from the general average, being higher in its content of fat and protein, and lower in lactose. The value for calcium is remarkably low. The first analysis of Laxa is stated to be of colostrum and agrees in its general characteristics with ours. The content of protein is very high.

Two useful comparisons may be made. It is interesting to compare fox milk with that of animals closely related zoologically. There are, however, very few analyses in the literature of the milk of animals other than domestic. The dog belongs to the

TABLE I
Analysis of Foz Milk

	Sample 1	Sample 2	Sample 2 (Laxa)	Sample 3	Sample 1 (Laxa)	Average	Method
Period of lactation, days.....	28	28	35	3	Colostrum	Late	Pycnometer
Sp. gr.....	1.035	1.035		1.028		per cent	
Water.....	82.56	81.16	81.88	75.90	65.21	81.87	Dish evaporation
Total solids.....	17.44	18.84	18.12	24.10	34.79	18.13	"
" proteins.....	5.45	6.59	6.71	8.85	17.04	6.25	Kjeldahl ($N \times 6.37$)
Fat.....	6.83	6.64	5.42	11.61	12.25	6.30	Soxhlet
Lactose.....	4.47	4.11	5.11	1.16	3.96	4.86	Owen and Gregg (10)
Ash.....	1.00	1.04	0.88	1.05	1.54	0.96	Ignition
Calcium (CaO).....	0.392	0.279		0.003		0.308	Clark and Collip (4)
Phosphorus (P_2O_5).....	0.470	0.284		0.191		0.331	Fiske and Subbarow (5)

same family (Canidæ) and the cat to the same order (Carnivora). The other analyses in Table II are included for comparative purposes.

We have selected from the analyses of Abderhalden those of milk samples taken about 10 days after parturition. His figures for the dog are on individual animals but for the cat an average of four. Compared with our average of the milk of the fox after 4 weeks of lactation, the latter is slightly lower in protein, intermediate in fat, and similar in lactose and salt content.

Compared with the milk of the cow that of the fox is distinctly different and richer as regards protein, fat, and energy content

TABLE II
Composition of Milk of Other Animals

Animal	Sp. gr.	Water	Total solids	Total proteins	Fat	Lactose	Ash	Observers
		per cent	per cent	per cent	per cent	per cent	per cent	
Fox.....	1.035	81.87	18.13	6.25	6.30	4.56	0.96	Authors and Laxa (8)
Dog.....	1.035	75.44	24.56	11.17	9.57	3.09	0.73	Heineman (6)
"				8.3	10.61	3.07	1.32	Pröscher (11)
"				6.76	11.31	3.42		Abderhalden (2)
Cat.....		82.45	17.55	7.00	4.75	4.78	1.02	" (1)
Ewe.....	1.035	83.57	16.43	5.15	6.18	4.17	0.93	Heineman (6)
Sow.....	1.038	83.94	16.06	7.23	4.55	3.23	1.05	"
Cow.....	1.031	87.27	12.73	3.39	3.68	4.94	0.72	"

and, as will be shown below, calcium phosphate. It is not surprising on this basis alone that the fox puppy does not thrive on cow's milk. The milk of the ewe probably shows the greatest similarity.

A second mode of comparison is based on the relationship between rate of growth of sucklings and protein, calcium, and phosphate levels of the mother's milk, (Bunge (3), Pröscher (11), Abderhalden (1, 2)). Such a relationship would seem to hold in a number of cases but an inspection of the table given by Heineman reveals many exceptions. Moreover, such a limited conception of growth is not tenable at the present time. The fox doubles its

weight in about 5 days and hence the analysis of fox colostrum in an individual case would be necessary to answer the possibility of this relationship.

When we compare the composition of these different types of milk, however, as to the content of the ash in calcium and phosphate, the fact is established that the content of fox milk is relatively high. Table III has been constructed from figures given mostly by Abderhalden (2) and calculated on a percentage basis by us. The ash content of cow's milk was taken from Leach (9). The additive figure of calcium and phosphate becomes 74 per cent of the total ash for the fox and 86 per cent in one

TABLE III
Comparative Calcium and Phosphate Content of Milk

Animal	Ash	Calcium (CaO)		Phosphorus (P ₂ O ₅)	
		Milk	Ash	Milk	Ash
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Cow.....	0.70	0.140	20.0	0.170	24.2
Sheep.....	0.84	0.245	29.2	0.293	35.0
Pig.....	0.81	0.249	30.8	0.308	38.0
Dog.....	1.33	0.454	34.0	0.508	38.3
Fox.....	0.96	0.336	35.0	0.377	39.3
Rabbit.....	2.50	0.891	35.6	1.00	40.0
Fox 1.....	1.00	0.392	39.2	0.470	47.0

sample. The average figures are only suggestive of the general content because of the wide variation as shown in Table I.

We thus have a second reason why cow's milk is so unsuitable as food for the fox puppy. There is unfortunately no analysis of the ash of cat milk available for comparison. We are informed that the fox is very susceptible to rickets in captivity and that it is good practice to supplement the diet with cod liver oil and ground bone.

SUMMARY

The milk of the vixen has been found to have the following composition on an average of five animals: water 81.87, total solids 18.13, protein 6.25, fat 6.30, lactose 4.56, ash 0.96 per cent.

Calcium and phosphate comprise 74 per cent of the ash but the variation is wide. On one sample a figure of 86 per cent was obtained. A sample of colostrum has also been analyzed.

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